

Multi-target inhibition by four tandem shRNAs embedded in homo- or hetero-miRNA backbones

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Abstract. The functional influence of microRNA (miRNA) backbone selection remains unclear with respect to multiplexing miRNA-based short hairpin RNAs (shRNAmiRs), due to a lack of comparative studies. To this end, a pair of shRNAmiR tetramers were designed in the present study that targeted four genes with a shared miR30a backbone (homo-BB) or four miRNA backbones (hetero-BB). A PBLT⁺ 293A cell line overexpressing four targets was established, which permitted simultaneous dissection of individual gene knockdown. Multi-target inhibition was confirmed by a decrease in positive cell populations of the relative gene and mean fluorescence intensities, with almost comparable activities of homo- and hetero-BB tetramers. Of note, this multi-inhibition was sustained over a 1-month period, with no notable difference, particularly in the late-phased inhibitory effects between homo- and hetero-BB tetra-shRNA miRs. These preliminary data may indicate little influence of scaffold substitution in the functionalities of multiplexed shRNAmiRs and little recombination-depleted risk of repetitively adopting the same miRNA backbone in this artificial *in vitro* system. More comparative studies are further required to explore extended repertoires of scaffold-paralleled multi-shRNAmiRs in more physiologically relevant models.

Introduction

The biological mechanism underpinning microRNA (miRNA) biogenesis allows for the understanding of the incorporation of

short hairpin RNAs (shRNA) targeting genes of interest into natural miRNA backbones (BBmiR). Chimeric miRNA-based shRNAs (shRNAmiR), driven by RNA polymerase II promoters, mimic primary miRNA (pri-miRNA) function and are processed via intrinsic small RNA biogenesis machinery without perturbation of endogenous miRNA homeostasis (1,2). A variety of shRNAmiRs have been under preclinical investigation in the context of various diseases (3).

Multi-target inhibition is a major concern for shRNAmiR design, especially in the case of infectious settings. To date, there have been several reports examining multiplexing approximately 2-7 shRNAmiRs in a single transcript with either the same BBmiR (homo-BB) or different BBmiRs (hetero-BB). Hetero-BB multiplexed shRNAmiRs are reconstituted based on authentic polycistronic miRNA clusters (4-6), as well as artificial tandem-arrayed miRNAs (7), and typically show additive antiviral properties compared to their mono-counterparts. However, homo-BB multiplexed shRNAmiRs commonly adopt the backbone of either miR30 (8-13) or miR155 (14-17) to accommodate the same or different shRNAs, targeting one or more genes, yielding inconsistent results regarding efficacy and stability of the homo-BB scaffold. Little is known regarding the impact of backbone selection on multi-shRNAmiR construction due to a lack of comparative studies of both hetero-BB and homo-BB.

The present study reports a paralleled design of tetra-shRNAmiRs constructed in hetero-BB and homo-BB simultaneously. Structural composition was based on several considerations. Firstly, it is known that more than four shRNAmiR cassettes in tandem result in less efficient knockdown when using C-terminal firefly luciferase (Fluc)-specific shRNAmiR as a functional readout (16). Therefore, a tetrameric approach was chosen. Secondly, in the tetramer, four shRNAmiRs were designed to target different genes [programmed cell death protein 1 (PD1), B- and T-lymphocyte attenuator (BTLA), lymphocyte activation gene 3 (LAG3) and T cell immunoglobulin mucin 3 (TIM3)] in order to distinguish individual shRNAmiR-related inhibition separately. Thirdly, miR30a, miR16-1, miR20a and miR122 were selected to construct the hetero-BB tetramer based on a previous study of multiplexing seven hetero-BB shRNAmiR (7). It was reported that when a shRNAmiR heptamer was processed to produce separate shRNAmiRs, the four selected miRNAs above

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exhibited higher processing efficiencies than the other three. In the present study, we used the modified miR-E version of miR30a, with enhanced processing and knockdown abilities, for the construction of both hetero-BB and homo-BB (18). Importantly, although no positional effects of shRNAmiRs as components in the multiplex have been reported (7,10,16), our study maintained the exact shRNAmiR alignment between homo- and hetero-BB tetramers, with their multi-target inhibition quantified and compared in an artificial cell model that co-overexpressed four target genes.

Materials and methods

Mono- and tetra-shRNAmiR constructs. Four or five shRNA sequences for each target gene were inserted into the indicated BBmiRs, generating four sets of mono-shRNAmiRs (Table I). Tetra-shRNAmiRs were designed by multiplexing four mono-shRNAmiRs (Table II). All mono- and tetra-shRNAmiR genes were synthesized (AuGCT Corp., Beijing, China) and cloned into pEGFP-C2 vector (Clontech Laboratories, Mountain View, CA, USA) to generate pEGFP-shRNAmiRs for transient transfection. Tetra-shRNAmiRs and Enhanced Green Fluorescent Protein (EGFP) were also cloned into pLVX-IRES-Puro (Clontech Laboratories) to produce pLVX-EGFP-tetra-shRNAmiR-IRES-Puro for lentivirus preparation.

Preparation of lentiviruses carrying tetra-shRNAmiRs. pLVX-EGFP-tetra-shRNAmiR-IRES-Puro was co-transfected into 293T cells with the helper plasmids pSPAX2 and pMD2.G (plasmids 12259 and 12260; Addgene Inc., Cambridge, MA, USA). At 72 h post-transfection, recombinant lentiviruses were harvested and adjusted to 1×10^6 PFU/ml. Lentiviral transduction was performed at a multiplicity of infection (MOI) of 1 in multi-target-overexpressing 293A cells (Fig. 1).

Establishment of multi-target-overexpressing cells. BTLA, PD1, TIM3 and LAG3 genes were purchased from Sino Biological Inc. (Beijing, China). Target-encoding lentiviral vectors were constructed by insertion of BTLA into pLenti6.3/V5-DEST (Invitrogen, Carlsbad, CA, USA), PD1 into pLVX-IRES-Puro (Clontech Laboratories), TIM3 into pLVX-IRES-Hyg (Clontech Laboratories) and LAG3 into pLVX-IRES-Neo (Clontech Laboratories). Recombinant vectors were co-transfected into 293T cells with pSPAX2 and pMD2.G for lentivirus production. The resultant lentiviruses were sequentially transduced into 293A cells, followed by selection of drug-resistant colonies with 10 μ g/ml blasticidin for BTLA expression, 0.25 μ g/ml puromycin for PD1 expression, 500 μ g/ml hygmycin for TIM3 expression and 1,000 μ g/ml G418 for LAG3 expression. Each selection lasted for 10 days, and finally multi-target-overexpressing 293A cells were obtained and identified by flow cytometry.

Flow cytometry. Target inhibition was quantified by flow cytometry via measuring downregulation of surface target proteins. Monoclonal antibodies used herein included phycoerythrin (PE)-conjugated anti-human PD1 and BTLA (BioLegend, San Diego, CA, USA), and PE-conjugated anti-human LAG3 and TIM3 (eBioscience, Inc., San Diego, CA, USA). After

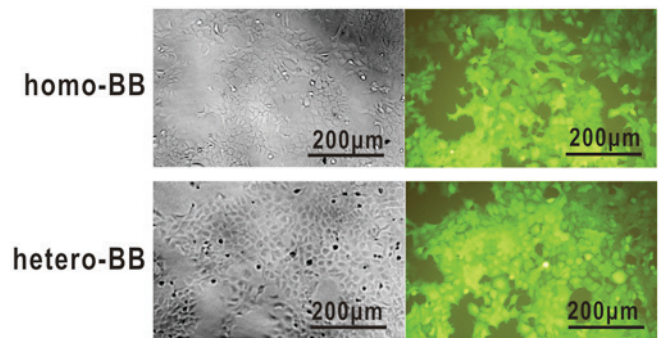


Figure 1. Transduction of tetra-shRNAmiRs visualized by EGFP fluorescence. Bright field and fluorescent images are shown. sh, short hairpin; miR, microRNA; EGFP, Enhanced Green Fluorescent Protein.

incubation on ice with the pertinent antibodies for 30 min, stained cells were analyzed by FACSCalibur using Cell-Quest software (both BD Bioscience, San Diego, CA, USA).

Quantitative real-time PCR (qRT-PCR). To detect individual siRNAs generated from tetra-shRNAmiRs, 500 ng of total RNA was extracted by Trizol (Invitrogen), followed by qRT-PCR using miScript PCR Starter Kit (Qiagen, Hilden, Germany). miScript universal primer was used in combination with the following specific primers: 5'-acgtgc-taaactggtaccgcat-3' (for siPD1-257); 5'-agaactagaatgccctgt gaaa-3' (for siBTLA-163); 5'-accatcaccacttagcggaag-3' (for siLAG3-651); 5'-agttgtcgtttgcaatgccat-3' (for siTIM3-883). The average cycle threshold values of mature siRNAs were normalized to that of U6 small nuclear RNA (snRNA).

Statistical analysis. Quantitative data are presented as mean \pm standard deviations (SD) from independent experiments in triplicate. Differences were tested for significance by ANOVA using SPSS 15.0. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BBmiR selection affects the efficacy of mono-shRNAmiRs. To ensure the efficacy of mono-shRNAmiR design, four sets of shRNA sequences embedded in the indicated BBmiRs were first examined for their ability to knockdown their related target genes. A co-transfection-based cell model was used to directly quantify shRNA-mediated downregulation of surface target proteins by flow cytometry analysis. As shown in Fig. 2A, shPD1-257, shBTLA-163, shLAG3-651 and shTIM3-884 were selected for further experiments.

Next, several shRNAs were assessed in combination with different BBmiRs. The resultant mono-shRNAmiRs were examined as to whether BBmiR substitution could interfere with a particular shRNA function. As evidenced by Fig. 2B, shBTLA-163-BBmiR30a led to a marked increase in BTLA inhibition by $27.8 \pm 3.71\%$ compared with its BBmiR20a counterpart. Similarly, in the case of shLAG3-651 and shTIM3-884, their BBmiR30a chimeras increased LAG3 inhibition by $10.93 \pm 5.05\%$ and TIM3 inhibition by $14.20 \pm 6.29\%$, respectively, compared with their BBmiR122 and BBmiR16-1 chimeras. These results suggest that BBmiR

Table I. Oligonucleotide sequences of mono-shRNAmiRs. Target-complementary sequences are underlined.

Target	shRNAmiRs	5'-3' Sequence
PD1	shPD1(257)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagctgctaaactggtaccgcattagtgaagccacagatgtaatgcg gtaccagtttagcaccggtgcctactgcctcggacttcaaggggctagaattc
	shPD1(458)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagacaggcgagatcaaagagagtagtgaagccacagatgtactctc tttgatctgcgccttggtgcctactgcctcggacttcaaggggctagaattc
	shPD1(746)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagaggttccagtggcgagagaatagtgaagccacagatgtattctctc gccactggaatccgtgcctactgcctcggacttcaaggggctagaattc
	shPD1(800)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagacggaggtatgccaccattagtgaagccacagatgtaattggtg gcatactccgtctgcgtgcctactgcctcggacttcaaggggctagaattc
	shPD1(818)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagactgtcttcttagcgggaattagtgaagccacagatgtaattccgc taggaaagacaatggtgcctactgcctcggacttcaaggggctagaattc
	shPD1(NC)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcgaatgccgacgccggaatatagtagtgaagccacagatgtagctatat tccggcgtcggcatgtgcctactgcctcggacttcaaggggctagaattc
BTLA	shBTLA(085)-BBmiR20a	ctcgaggctctatctgatgtgacagcttctgtagcacccatgggaaagaatcatgtgattgtttatgttatcacatgattcattc ccatgcgtactgctagctgtagaactccagcttcggcgaaattc
	shBTLA(152)-BBmiR20a	ctcgaggctctatctgatgtgacagcttctgtagcacggagatccctttgaactagaattgttttagttatattctagttaacggga tctcgtactgctagctgtagaactccagcttcggcgaaattc
	shBTLA(163)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagagatccctttgaactagaattagtgaagccacagatgtaattctagt tcaaaggatctcgtgcctactgcctcggacttcaaggggctagaattc
	shBTLA(163)-BBmiR20a	ctcgaggctctatctgatgtgacagcttctgtagcactgaactagaatgccctgtgaaatgttttagttatttcacagggcaatc tagttccgtactgctagctgtagaactccagcttcggcgaaattc
	shBTLA(332)-BBmiR20a	ctcgaggctctatctgatgtgacagcttctgtagcacgtcataccgctgttctgcaaatgttttagttatatttcagaaacaccg gtatgaggctactgctagctgtagaactccagcttcggcgaaattc
	shBTLA(NC)-BBmiR20a	ctcgaggctctatctgatgtgacagcttctgtagcacgattctatcagtcgtgcatactgttttagttatggtatgcacgacagat agaatggtactgctagctgtagaactccagcttcggcgaaattc
LAG3	shLAG3(651)-BBmiR122	ctcgagcgtggctacagagtttcttagcagagctgccatcaccacttagcggaaagtgtctaaactatcttccgctaaga gggtatggctagctactgctaggaatccttccctcgataagaattc
	shLAG3(651)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcagaccatcaccacttagcggaaagtgtgaagccacagatgtactttccgc taagtgggtatgggtgcctactgcctcggacttcaaggggctagaattc
	shLAG3(747)-BBmiR122	ctcgagcgtggctacagagtttcttagcagagctgtcaacgtctccatcatgtataatgtctaaactatttatacatgatgcag acgttgcgtactgctaggaatccttccctcgataagaattc
	shLAG3(999)-BBmiR122	ctcgagcgtggctacagagtttcttagcagagctggccatcatcctgcaggaacatgtctaaactattgttctgcaga aggatattgggtagctactgctaggaatccttccctcgataagaattc
	shLAG3(1102)-BBmiR122	ctcgagcgtggctacagagtttcttagcagagctggctttgtgaggtgactccagtatgtctaaactatttgcagtcacgt caciaaggtagctactgctaggaatccttccctcgataagaattc
	shLAG3(NC)-BBmiR122	ctcgagcgtggctacagagtttcttagcagagctgggaccgctagaacgctcactaatgtctaaactattttagtgagcgttg tagcggctgtagctactgctaggaatccttccctcgataagaattc
TIM3	shTIM3(201)-BBmiR16-1	ctcgaggctcttatgatagcaatgtcagcagtgctgcagcagctgatgaaagggttaagattctaaaattatattcccttt catcgtcctgaggagtaagggtgaccatactctacagttgtgtgaattc
	shTIM3(539)-BBmiR16-1	ctcgaggctcttatgatagcaatgtcagcagtgctgcgaatgagttacgggactctattaagattctaaaattattagagtc ccgtacctcattgggagtaagggtgaccatactctacagttgtgtgaattc
	shTIM3(750)-BBmiR16-1	ctcgaggctcttatgatagcaatgtcagcagtgctgcgaatgcagtagcagagggaattaagattctaaaattatttccctct gctagtgcatttggagtaagggtgaccatactctacagttgtgtgaattc
	shTIM3(884)-BBmiR16-1	ctcgaggctcttatgatagcaatgtcagcagtgctggtgtcgtttgcaatgccatttaagattctaaaattatattgacattg caacgcgacaacgagtaagggtgaccatactctacagttgtgtgaattc
	shTIM3(884)-BBmiR30a	ctcgagaaggtatattgctgttgacagtgcgcaggtgtcgtttgcaatgccattagtgaagccacagatgtaattggcattg caaagcgacaacgtgcctactgcctcggacttcaaggggctagaattc
	shTIM3(NC)-BBmiR16-1	ctcgaggctcttatgatagcaatgtcagcagtgctgcagctctatcgttaccggtgttcttaagattctaaaattatgaacaccg taactatagacgtgagtaagggtgaccatactctacagttgtgtgaattc

PD1, programmed cell death protein 1; BTLA, B- and T-lymphocyte attenuator; LAG3, lymphocyte activation gene 3; TIM3, T cell immunoglobulin mucin 3; sh, short hairpin; miR, microRNA.

Table II. Oligonucleotide sequences of tetra-shRNAmiRs. Bold motifs in each sequence are recognized by *Xho* I, *Spe* I, *Xba* I, *Not* I and *Eco*R I in turn.

Oligonucleotides	5'-3' Sequence
homo-BB tetra-shRNAmiR	ctcgaga aggtatattgctgttgacagtgagcgacgtgctaaactggtaccgcattagtgaagccacagatgtaatcggtaccag tttagcacggtgcctactgcctcggacttcaaggggctata actag tacaaggtatattgctgttgacagtgagcgagaactagaatgc cctgtgaaatagtgaaagccacagatgtatttcacagggcattctagttcgtgcctactgcctcggacttcaaggggctaat gtctaga aaacgaaggtatattgctgttgacagtgagcgaccatcaccacttagcggaaagtagtgaagccacagatgtacttccgctaagtgg tgatgggtgcctactgcctcggacttcaaggggctatt gcggcgcg caacaaggtatattgctgttgacagtgagcgagttgctgctttg caatgccattagtgaagccacagatgtaatggcattgcaagcgacaacgtgcctactgcctcggacttcaaggggct agaattc
hetero-BB tetra-shRNAmiR	ctcgaga aggtatattgctgttgacagtgagcgacgtgctaaactggtaccgcattagtgaagccacagatgtaatcggtac cagtttagcacggtgcctactgcctcggacttcaaggggctata actag tacgtctatctgtagtgcagcttctgtagcacgtcatac cgctgttctgcaaattgttagttatattgcagaacaccggatgaggtactgtagctgtagaactccagcttcggcat gtctagaaaac gcgtggctacagagtttcttagcagagctgccatcaccacttagcggaaagtgtctaaactatcttccgtaagaggtgatggc tagctactgtaggaatcttccctcgataatt gcggcgcg caacgctcttatgatagcaatgtagcagctgcctggtgctgctttg caatgccattgaagtctaaattatattgcatgcaacgcgacaacgagtaaggttgaccatactctacagttgt gaattc

Sh, short hairpin; miR, micro RNA.

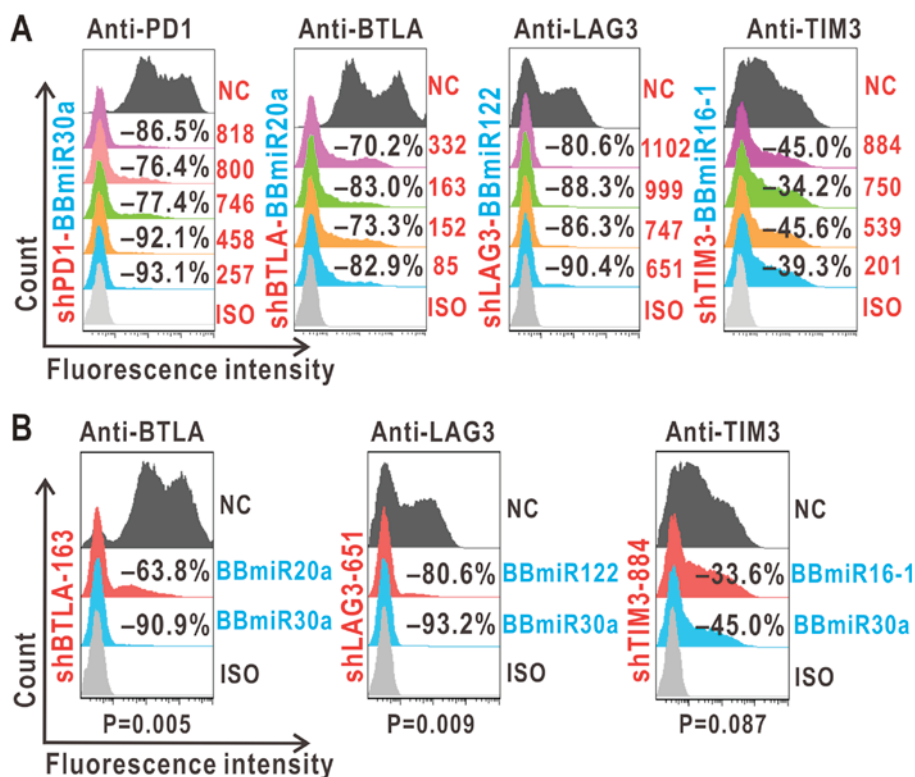


Figure 2. Identification of mono-shRNAmiRs targeting PD1, BTLA, LAG3 and TIM3. (A and B) shRNA selection with the indicated BBmiR. (A) Forty-eight-hour co-transfection of target genes and pEGFP-shRNAmiRs led to diminished target protein levels in 293A assayed by flow cytometry. (B) Silencing effects of different BBmiRs as compared with BBmiR30a at 36 h post-transfection. Negative percentages denote inhibition against scrambled shRNA controls (NC). ISO, isotype controls; PD1, programmed cell death protein 1; BTLA, B- and T-lymphocyte attenuator; LAG3, lymphocyte activation gene 3; TIM3, T cell immunoglobulin mucin 3; sh, short hairpin; miR, microRNA; BBmiR, miRNA backbones.

selection contributes, at least in part, to mono-shRNAmiR function.

Limited effect of BBmiR substitution on tetra-shRNAmiR function. Tetra-shRNAmiRs were generated by tandem fusion of four mono-shRNAmiRs targeting PD1,

BTLA, LAG3 and TIM3 (Fig. 3). To determine whether BBmiR substitution affected the function of multiplexed shRNAmiRs, two paralleled tetra-shRNAmiRs were designed with exactly the same targeting alignments but distinct backbones to generate homo-BB tetra-shRNAmiR with only BBmiR30a and hetero-BB tetra-shRNAmiR with

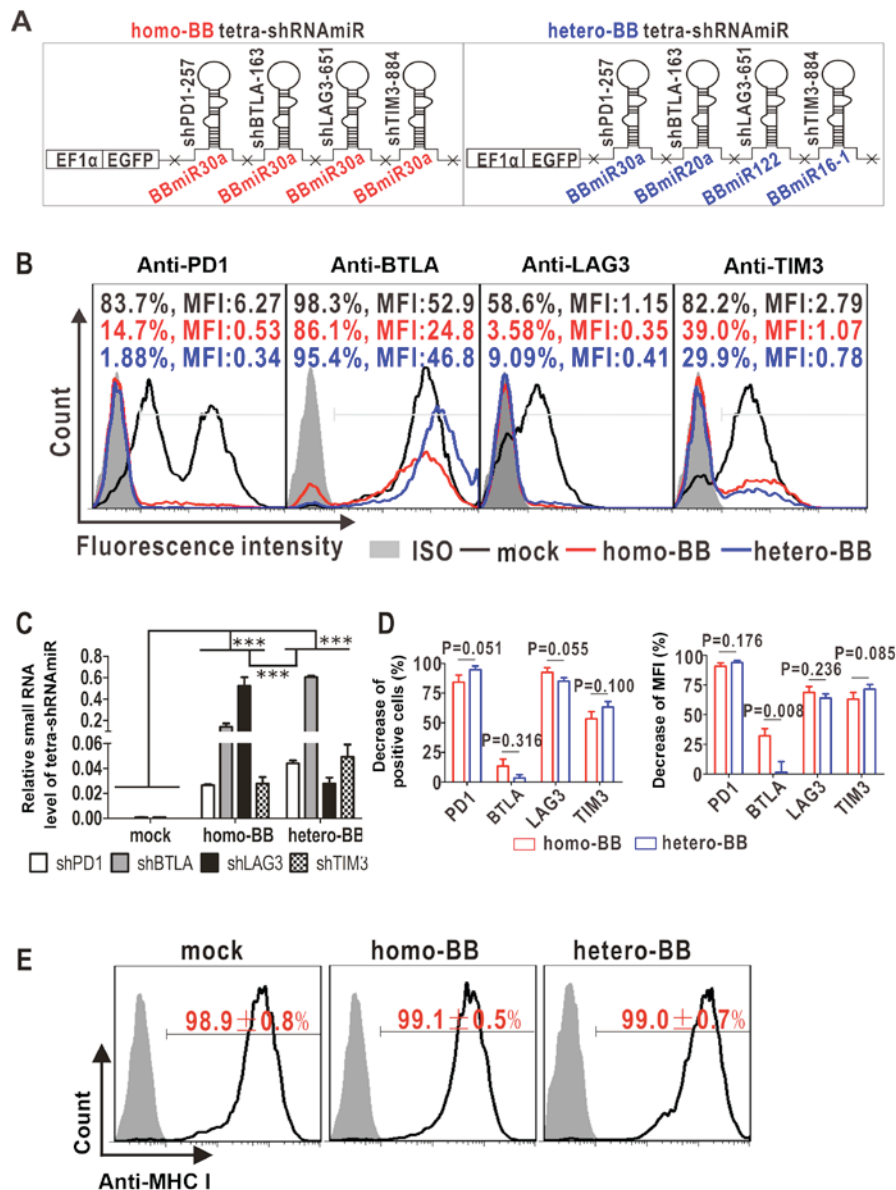


Figure 3. Multi-target effect of tetra-shRNAiRs on the established 293A cells simultaneously overexpressing PD1, BTLA, LAG3 and TIM3 (designated as PBLT⁺ 293A). (A) Schematic diagram of tetra-shRNAiRs with homo-or hetero-BBmiRs. X, restriction enzyme sites. (B) Target inhibition by flow cytometry at 4 days post-infection. A set of representative histograms are shown, with cell percentages and mean fluorescence intensities (MFI) of immunostained cells marked. ISO, isotype controls. (C) Normalized small RNA levels of tetra-shRNAiRs to U6 at 4 days post-infection by quantitative RT-PCR. ***P<0.001. (D) Statistical analyses of the decrease in both cell percentages and MFI against the void vector control (mock) from three independent experiments. (E) Undetected off-target effect of tetra-shRNAiRs on an irrelevant antigen (MHC I). Isotype controls are filled in grey. PD1, programmed cell death protein 1; BTLA, B- and T-lymphocyte attenuator; LAG3, lymphocyte activation gene 3; TIM3, T cell immunoglobulin mucin 3; sh, short hairpin; miR, microRNA.

four different BBmiRs (Fig. 3A). For functional comparison of these distinct backbone-based tetra-shRNAiRs, an artificial cell model overexpressing all the targets was established and referred to as PBLT⁺ 293A. Selection with sequential drugs gave rise to high-level co-expression of PD1, BTLA, LAG3 and TIM3 (Fig. 4). Of note, when cultured without drugs for two months, PBLT⁺ 293A cells stably expressed BTLA, while the expressions of other three target genes gradually declined with prolonged culture (Fig. 3B, black lines). Given this slowly declining trend of target gene expression, non-drug-treated mock-transfected control cells were monitored at all time points and relative target inhibition over the mock was calculated to exclude intergroup variations.

Transduction of tetra-shRNAiRs resulted in processing and production of individual mature siRNAs in PBLT⁺ 293A cells, as detected by quantitative RT-PCR (Fig. 3C). Construction with the hetero-BB contributed to more abundant generation of small RNAs targeting PD1, BTLA and TIM3, whereas the homo-BB design was associated with lower processing efficiencies, presumably due to competition for small RNA biogenesis machinery. Unexpectedly, LAG3-targeted siRNA production predominated in the homo-BB group rather than the hetero-BB group, indicating a more potentiated processing capability of shLAG3-BBmiR30a than that of shLAG3-BBmiR122.

Multi-target effects of the two tetra-shRNAiRs were further compared. As shown in Fig. 3B and D, tetramer-transduced

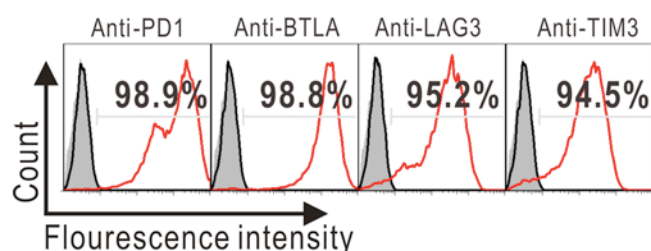


Figure 4. Establishment and identification of PBLT⁺ 293A cells by flow cytometry, with the specific antibodies lined in red and the isotypes filled in grey. Parental 293A, lined in black, served as a negative control. PD1, programmed cell death protein 1; BTLA, B- and T-lymphocyte attenuator; LAG3, lymphocyte activation gene 3; TIM3, T cell immunoglobulin mucin 3.

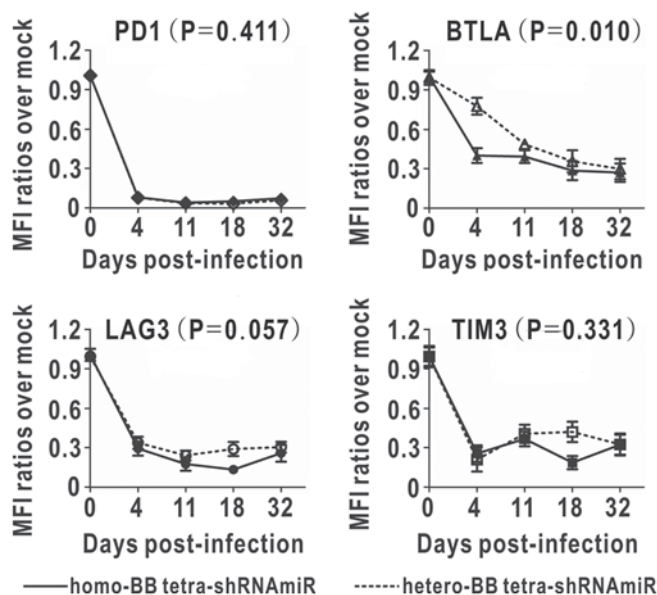


Figure 5. Sustained target inhibition of tetra-shRNAmiRs in PBLT⁺ 293A cells detected by flow cytometry. MFI ratios (% of the mock) are presented to exclude interference from gradual loss of exogenous targets in long-term cultured cells. Statistical analyses were performed by two-way ANOVA. PD1, programmed cell death protein 1; BTLA, B- and T-lymphocyte attenuator; LAG3, lymphocyte activation gene 3; TIM3, T cell immunoglobulin mucin 3; sh, short hairpin; miR, microRNA.

PBLT⁺ 293A cells exhibited potent inhibition on PD1, LAG3 and TIM3 in both groups, as evidenced by the remarkable parallel decreases in cell percentages and mean fluorescence intensities (MFI), and no significant difference resulted from BBmiR substitution. In contrast, BTLA inhibition was relatively weak in both tetramers (Fig. 3B and D). This may be in part attributed to the significant expression of BTLA in non-drug-treated mock controls, which exhibited extremely high MFI of 56.4 ± 5.9 in $97.6 \pm 1.1\%$ BTLA-positive cells, in contrast to the much weaker expression of the other three targets with MFI below 10 (Fig. 3B, black lines and black numbers). Thus there is a possibility that tetra-shRNAmiRs were insufficient to markedly downregulate highly expressed BTLA. Interestingly, homo-BB-tetramer transductants exhibited stronger BTLA inhibition than hetero-BB counterparts, with significant differences in MFI decrease (Fig. 3D). No off-target effects were observed as evidenced by unaltered expression of an irrelevant antigen (MHC I) after infection of tetra-shRNAmiRs (Fig. 3E).

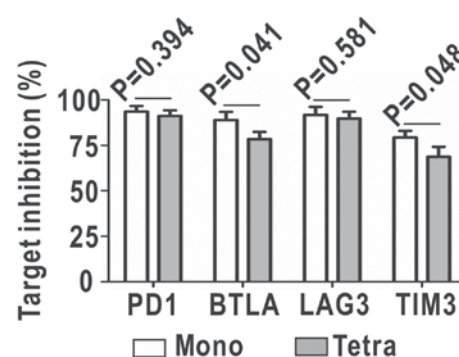


Figure 6. Comparison of inhibitory effects of homo-BB tetra-shRNAmiR and the corresponding mono-shRNA-BBmiR30a. 293A cells were transiently co-transfected with the target genes and pEGFP-shRNAmiRs. Inhibition rates against the mock were analyzed by flow cytometry at 48 h post-transfection. PD1, programmed cell death protein 1; BTLA, B- and T-lymphocyte attenuator; LAG3, lymphocyte activation gene 3; TIM3, T cell immunoglobulin mucin 3; sh, short hairpin; miR, microRNA.

Taken together, in this particularly tested tetra-shRNAmiR formulation, BBmiR substitution had limited effects on overall functionality.

Sustained functionality of homo- and hetero-BB tetra-shRNAmiRs. To compare long-term inhibitory effects of the two tetramers, MFI decreases of multi-targets were monitored for one month (Fig. 5). Simultaneous downregulation of four targets were observed in both groups. Inhibition rate of PD1 was over 90%, and those of BTLA, LAG3 and TIM3 ranged from 67 to 74% at the final endpoint. There was no intergroup difference shown in persistent inhibition of PD1, LAG3 and TIM3, in contrast to BTLA inhibition, which was significantly enhanced with homo-BB structure. The enhancement of BTLA inhibition in the homo-BB group peaked on day four post-infection when comparing with the hetero-BB group. On the whole, long-term inhibitory effects of tetra-shRNAmiRs with substituted BBmiRs were almost comparable at the end of one month.

Inhibition efficiency of homo-BB tetra-shRNAmiR. In order to verify whether homo-BB tetra-shRNAmiR would reduce the efficiency of each shRNA, we co-transfected target genes together with mono-shRNAmiRs or homo-BB tetra-shRNAmiR into 293A cells, and directly quantified shRNA-mediated downregulation of surface target proteins by flow cytometry. As shown in Fig. 6, knockdown efficiencies on PD1 and LAG3 were comparable between mono-shRNAmiRs and the tetra-shRNAmiR. Despite a slight decrease in downregulation of BTLA and TIM3 by $\sim 10\%$, the tetra-shRNAmiR still exhibited potent inhibition $\sim 70\%$. Therefore, the BBmiR-shared strategy of multiplexing shRNAmiR may likely result in a little lower efficiencies of multi-target inhibition, but is functionally competent and conveniently manipulated.

Discussion

Recent insights into tandem shRNAmiR strategies have accumulated out of separate studies with either homo-BB or hetero-BB alone. Our design of BBmiR-substituted

tetra-shRNAmiRs, to the best of our knowledge, is the first attempt to comparatively explore the contribution of BBmiR scaffolds to multiplexed shRNAmiR functions. To date, there has been only one report on comparison of homo- and hetero-dimers of natural miRNAs (19), which differs from our study in several ways. Firstly, no artificial shRNA sequences were exchanged into dimerized miRNAs, failing to offer a structure-function relationship of shRNAmiR multiplexes. Secondly, several cell models were developed by introducing natural miRNA-complementary motifs into 3'-UTR of GFP reporter, hardly affording simultaneous assessment of both miRNA functions. In contrast, our study is characterized by combining shRNA-adapted artificial miRNA tetramer with strictly paralleled BBmiRs, and by targeting four different genes to validate individual knockdown effect at the same time.

Intriguingly, two seemingly paradoxical findings were highlighted by our study. The first was that despite superior functionalities of miR30a-backed mono-shRNAmiR, the homo-BB tetramer exerted multi-inhibitory effects almost similar to the hetero-BB tetramer. One of possible interpretations was a lowered individual threshold of expression and processing due to shRNAmiR multiplexing. As evidenced by a previous study on seven-chained hetero-BB shRNAmiRs, the four BBmiRs that we chose for our study decreased individual processing efficiencies by 70-80% compared with their mono-counterparts (7). These sharply narrowed assay windows could reduce functional differences between the two tetramers. Another underlying possibility was potent target inhibition beyond a linear range. This was indicated by loss of early-phased difference in BTLA downregulation when reaching late-phase inhibition as high as 70% (Fig. 5).

The second confounding issue was inconsistency between individual mature siRNA levels derived from tetra-shRNAmiRs and consequent knockdown efficiencies. Technically, we used the same primers in qRT-PCR for each pair of backbone-grafted shRNAmiRs targeting the same genes, thus amplified products of exactly the same mature siRNAs would be quantitatively analyzable for comparison of tetramer processing. In this study, processing preference was generally observed in the hetero-BB tetramer, as evidenced by higher production of siPD1, siBTLA and siTIM3. Of note, the homo-BB tetramer, although challenged by resource competition, produced higher amounts of siLAG3, which might be attributed to an unknown processing bias. However, such differences in mature siRNA levels did not functionally correlate, given almost comparable inhibitory activities between the two tetramers. Explanations behind this might involve PCR-based limitations of magnifying intergroup variations and failing to discern incorrectly processed products with minor alterations. To overcome this problem, deep sequencing may be a more reliable approach to confirm processing accuracy (7), and more efforts are needed to underpin the mechanisms behind this discrepancy.

Lentiviral risks of recombination-mediated deletion have been documented sporadically for both homo-BB-polymerized shRNAmiRs (12) and homo-dimerized natural miRNAs (19). However, extensive studies still confirm the validity of the sharing of the same BBmiR30 for shRNAmiR multiplexing, which functions as a co-inhibitor of different targets (8-10),

as well as additive inhibition against different sites of one target (11), and dosage-dependent inhibition against an identical site of one target (10). These findings are further supported by our current data on the one-month prolonged efficacy of the homo-BB tetra-shRNAmiR, which also provides the first evidence of feasibility of repetitively utilizing the modified backbone of the miR30a derivative miR-E. This shared backbone-based tetramerization did not substantially attenuate joint target inhibition, as shown by functional comparison with mono-shRNAmiRs in the co-transfection assay (Fig. 6).

In summary, this comparative study on BBmiR-paralleled shRNAmiR tetramers, by establishing a cell model to dissect target inhibition simultaneously, offered preliminary clues regarding scaffold influence on tetramer function. In our assay system, BBmiR substitution was not a major contributing factor, with nearly no marked functional difference between homo- and hetero-tetramers. Further efforts remain necessary with regards to the detailed validation of extended repertoires of scaffold-grafted shRNAmiR polymers in more physiologically relevant models.

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