

The analysis of dose response curve comes in useful for the assembly of multi-siRNAs expressing cassettes

Research Article

Laura Polisenò¹, Monica Evangelista¹, Mauro Giacca² and Giuseppe Rainaldi^{1,*}

¹Laboratory of Gene and Molecular Therapy, Institute of Clinical Physiology, CNR, Pisa, Italy;

²International Center for Genetic Engineering and Biotechnology, Trieste, Italy

***Correspondence:** Dr. Giuseppe Rainaldi, Laboratory of Gene and Molecular Therapy, Institute of Clinical Physiology, CNR, Area della Ricerca, Via Moruzzi, 1, 56124 Pisa, Italy; FAX: +39 050 3153327; Tel: +39 050 3153108; E-mail: g.rainaldi@ifc.cnr.it

Key words: simultaneous gene expression knock-down, competition among siRNAs, multi-shRNA vectors

Abbreviations: Dulbecco's modified Eagle's medium (DMEM); Fetal Bovine serum (FBS); Insulin-like growth factor I receptor (IGF-IR); multiple cloning site (MCS); platelet-derived growth factor receptor β (PDGF-R β); urokinase type plasminogen activator (uPA)

Received: 14 November 2005; Accepted: 8 March 2006; electronically published: March 2006

Summary

The construction of vectors that would allow the simultaneous expression of multiple siRNAs targeted against different genes is hampered by the competition between siRNAs. In this work, the simultaneous knock-down of four genes involved in smooth muscle cells activation, migration and proliferation was considered. We used the knock-down of EGFP reporter assay to evaluate the dose response curves of the four shRNA expressing plasmids. We found that each siRNA reached the highest activity (as evaluated from the plateau phase) with different kinetics (as evaluated from the KD). Due the specificity of KD, the mono-specific plasmids were tested against their targets by the addition of saturating amounts of each of the other shRNA-expressing plasmid. In this way, stronger from weaker shRNAs were distinguished and KD seemed to account for it. Moreover, when stronger shRNAs were assembled, the resulting plasmid was able to simultaneously transcribe active shRNAs genes. These results indicate that expression cassettes for different siRNAs having similar KD can be efficiently and rapidly assembled into multi-specific multi-siRNA plasmids. A practical correlate of these observations is that, in order to obtain effective multi-gene knock down, only siRNAs with similar inhibitory kinetics need to be delivered to the cells.

I. Introduction

Since the demonstration that synthetic siRNAs can be used to successfully knock-down gene expression, several efforts have been made to prolong their temporally limited activity. For this purpose, different RNA polymerase III-dependent siRNA expression cassettes have been developed and inserted into retroviral, lentiviral and adenoviral vectors (Brummelkamp et al, 2002). In this context, the possibility to construct vectors for the delivery of multiple siRNAs represents a further challenge in RNAi applications (Elbashir et al, 2002; Leirdal and Sioud, 2002; Anderson et al, 2003; Yu et al, 2003; Schuck et al, 2004). One of the concerns that arises when multiple siRNAs are present inside the cells is their possible competition for the RNAi machinery. Indeed, it has been reported that pools of active siRNAs targeted against

different regions of the same RNA do not always show additive/synergistic effects (Holen et al, 2002; Kawasaki et al, 2003; Hsieh et al, 2004) and that inactive siRNAs decrease the efficiency of active ones (McManus et al, 2002; Wunsche and Sczakiel, 2005). Hence, the construction of vectors that would allow either the expression of shRNA targeted against different regions of the same gene, in order to increase target gene knock-down efficiency, or the simultaneous expression of multiple siRNAs targeted against different genes is highly dependent on the competition between siRNAs. Insulin-like growth factor I receptor (IGF-IR), platelet-derived growth factor receptor β (PDGF-R β), urokinase type plasminogen activator (uPA) and α_v integrin genes are involved in the control of arterial of smooth muscle cell activation, proliferation and migration (Kopp and de

Martin, 2004). In order to achieve the simultaneous down-regulation of these genes as a therapeutic approach for prevention of arterial restenosis, it is crucial to know if active siRNAs targeted against these genes compete or not with to each other. We have already identified active siRNAs against these genes on the basis of functional asymmetry (Schwarz et al, 2003) and internal instability (Khvorova et al, 2003) and proved that they are active against their own targets (Poliseno et al, 2004). The reasons are that the antisense strand is loaded in RISC complex more efficiently if its stability is lower than that of sense strand, while a low stability in the middle of the antisense strand facilitates the mRNA cleavage.

However, these parameters seem inadequate to predict also if the selected siRNAs compete to each other, in that we found no clear relationship between them and competition. Titration experiments by using exogenously added siRNAs have already indicated that the efficacy of RNAi results from the equilibrium between the number of target mRNA molecules produced by the transcription

machinery and the number of molecules that are effectively cleaved (Elbashir et al, 2002; Holen et al, 2002). We reasoned that each siRNA challenged against its own target should generate a specific dose-response curve. This was true since we distinguished stronger (higher KD values) from weaker (lower KD values) shRNA. We also demonstrated that shRNA with similar dose-response curves can be assembled in a plasmid to give an effective multi-gene knock-down.

II. Materials and methods

A. shRNA expressing plasmids

The position of the siRNAs targeted against porcine genes (I, P, U and A siRNA) is shown in **Figure 1A**. Plasmids expressing the shRNAs under the control of the H1-RNA promoter (pI, pP, pU, pA pMCSpSUPER plasmids) are depicted in **Figure 1B**. They were constructed according to the procedure described in the legend of **Figure 4** (first-third step).

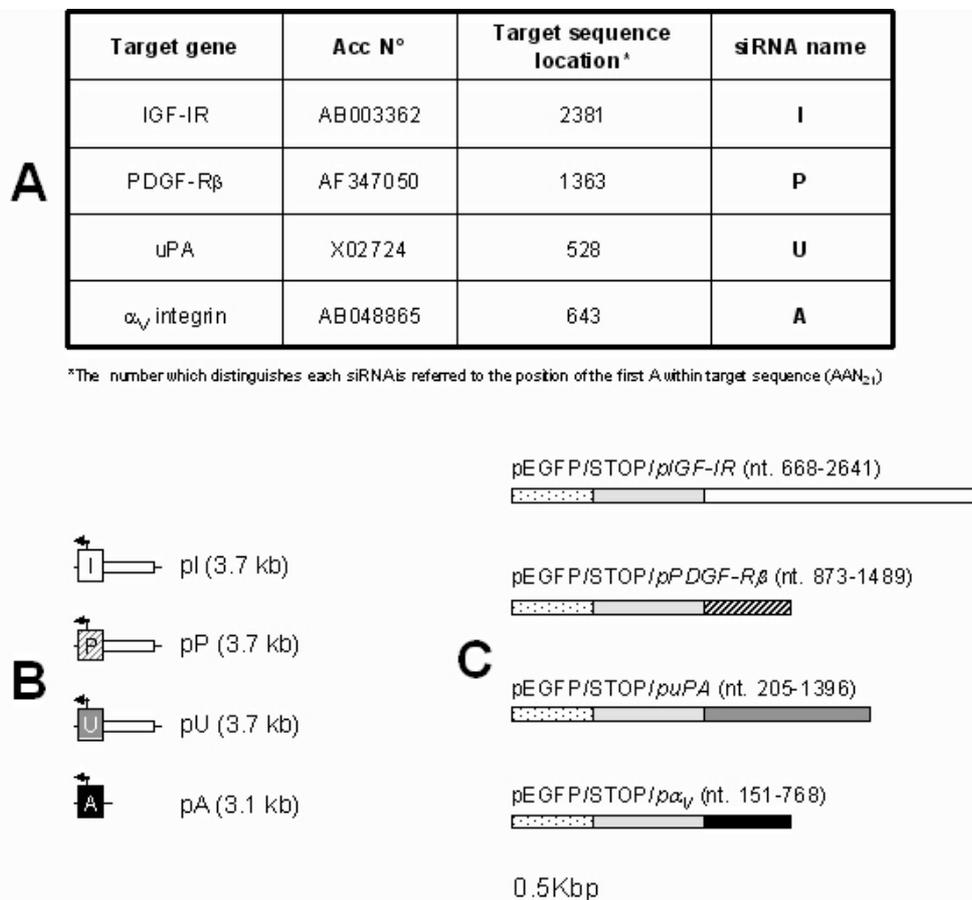


Figure 1. Activity of shRNA expressing plasmids. (A) Target genes involved in arterial restenosis (Kopp and de Martin, 2004), location of target sequence and siRNA nicknames. (B) Schematic representation of the 300 bp siRNA expression cassettes. pMCSpSUPER plasmids expressing I shRNA (pI), P shRNA (pP), U shRNA (pU) and A shRNA (pA) are depicted. The 600 bp spacer is reported on the right. Arrows indicate the direction of transcription. (C) Schematic representation of the reporter pEGFP-C1 hybrid plasmids used for the EGFP knock-down reporter assay. Indicated fragments of porcine genes were obtained by RT-PCR amplification from porcine coronary smooth muscle cells and cloned downstream of EGFP open reading frame in pEGFP-C1 plasmid. The forward primer contained a stop codon in order to ensure correct translation of the EGFP protein (Poliseno et al, 2004). Dotted box: CMV promoter; light gray box: EGFP ORF; white box: *pIGF-IR* porcine gene fragment; dashed box: *pPDGF-R β* porcine gene fragment; dark gray box: *puPA* porcine gene fragment; black box: α_v porcine gene fragment.

B. Reporter genes

To assess shRNA activity against their respective targets, we applied an already developed EGFP knock-down reporter assay (Poliseno et al, 2004). Briefly, a fragment of the target gene obtained by RT-PCR amplification from porcine coronary smooth muscle cells was cloned downstream of the EGFP open reading frame in pEGFP-C1 plasmid. The forward primer contained a stop codon in order to ensure translation of wt EGFP protein. Any shRNA targeting the resulting hybrid transcript determines a decrease in cellular fluorescence, which is then quantified by flow cytometry. The hybrid plasmids used as targets in the EGFP knock-down reporter assay are reported in **Figure 1C**.

C. Recipient cells and transfection

HEK293T human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium (DMEM) +10% Fetal Bovine serum (FBS) at 37°C in a humidified atmosphere containing 6% CO₂. The cells to be transfected were seeded at a density of 6x10⁵ cells/30-mm dish. After 24h, 40 pM hybrid plasmids were cotransfected in HEK293T cells with the indicated amounts of the appropriate shRNA expressing plasmid using Polyfect (Quiagen, Hilden,D) according to manufacturer's recommendations. At 36 h post transfection, fluorescence was measured by flow cytometry (FACScalibur, Becton Dickinson, San José, CA) using 10⁴ cells per sample.

D. Northern blotting

The pI, pP and pIP plasmids (40 pM) were transfected into HEK293T cells, as reported above. After 3 days, total RNA was extracted and analyzed by Northern blotting according to an established procedure (Czuderna et al, 2003). [³²P]-5'-end-labelled ssDNA oligonucleotides probes were used to detect hairpin I siRNA (5'-tctcttgaaggaaatgacagttctctcc-3'), and P siRNA (5'-tctcttgaagtcgagatcttgaccagc-3'). Cellular tRNA^{Val} (5'-gaacgtgataaccactacactacggaac-3') was used as a control. After PhosphorImager scanning, quantification of the radioactive

bands was performed using OptiQuant Acquisition and Analysis software.

III. Results

A. Dose response curves of shRNA-expressing plasmids

We tested the dose-response effect of different concentrations of the four single-copy shRNA-expressing plasmids against a fixed concentration of their respective targets. We observed that the maximal activity was reached at ~8 pM for all the four plasmids. It corresponded to a 83.9, 89.2, 78.2 and 84.2% decrease of EGFP fluorescence for pI, pP, pU and pA respectively (**Figure 2**).

Of interest, when analyzing the relative efficiencies of the four plasmids at lower concentrations, we observed that KD (the concentration at which 50% of the maximal activity of each plasmid was obtained) was 1.73, 2.19, 2.40 and 6.11 pM for pP, pI, pU and pA respectively (insert of **Figure 2**). These results highlight that each shRNA reached the highest activity (as evaluated from the plateau phase) with different kinetics (as evaluated from KD).

B. Competition between shRNAs

The efficacy of the mono-specific plasmids against their targets in the presence of saturating amounts of all the other siRNA-expressing plasmids is shown in **Figure 3**. The activity of pI against *pIGF-IR* reporter gene was reduced by 19% by pP, by 23% by pU and by 24% by pA. The activity of pP against its *pPDGF-Rβ* reporter gene was almost unaffected by the co-transfection of any of the

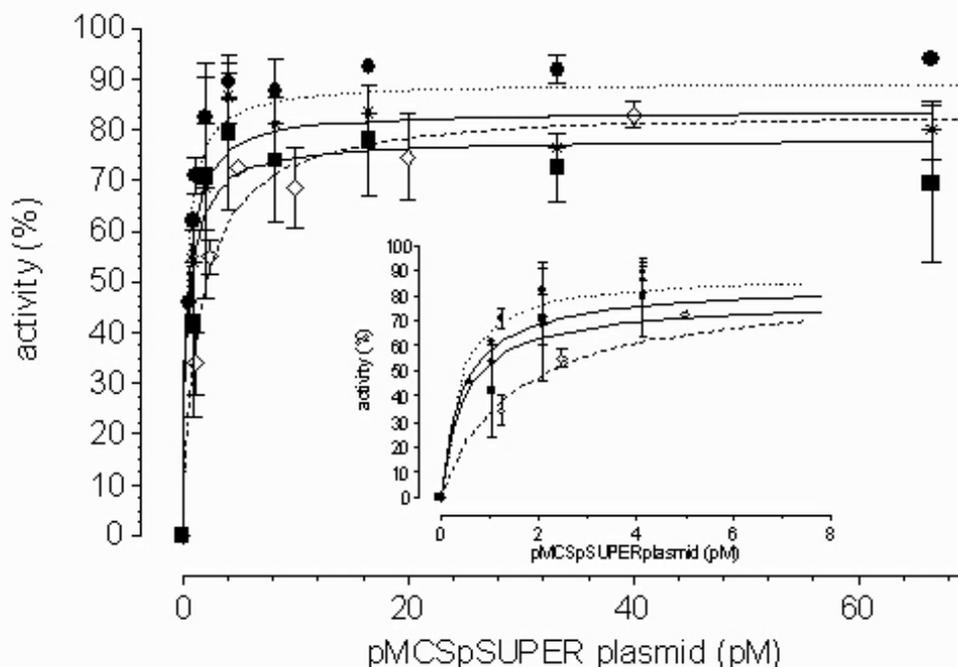


Figure 2. Dose response curves. 40pM hybrid plasmids were cotransfected in HEK293T cells with the indicated amounts of pI (■,-), pP (●,ααα), pU (▲,-) and pA (◇,-) plasmids. The mean ± SD of at least 3 independent experiments is reported. The 1 to 8pM concentration range is expanded in the inserted graph.

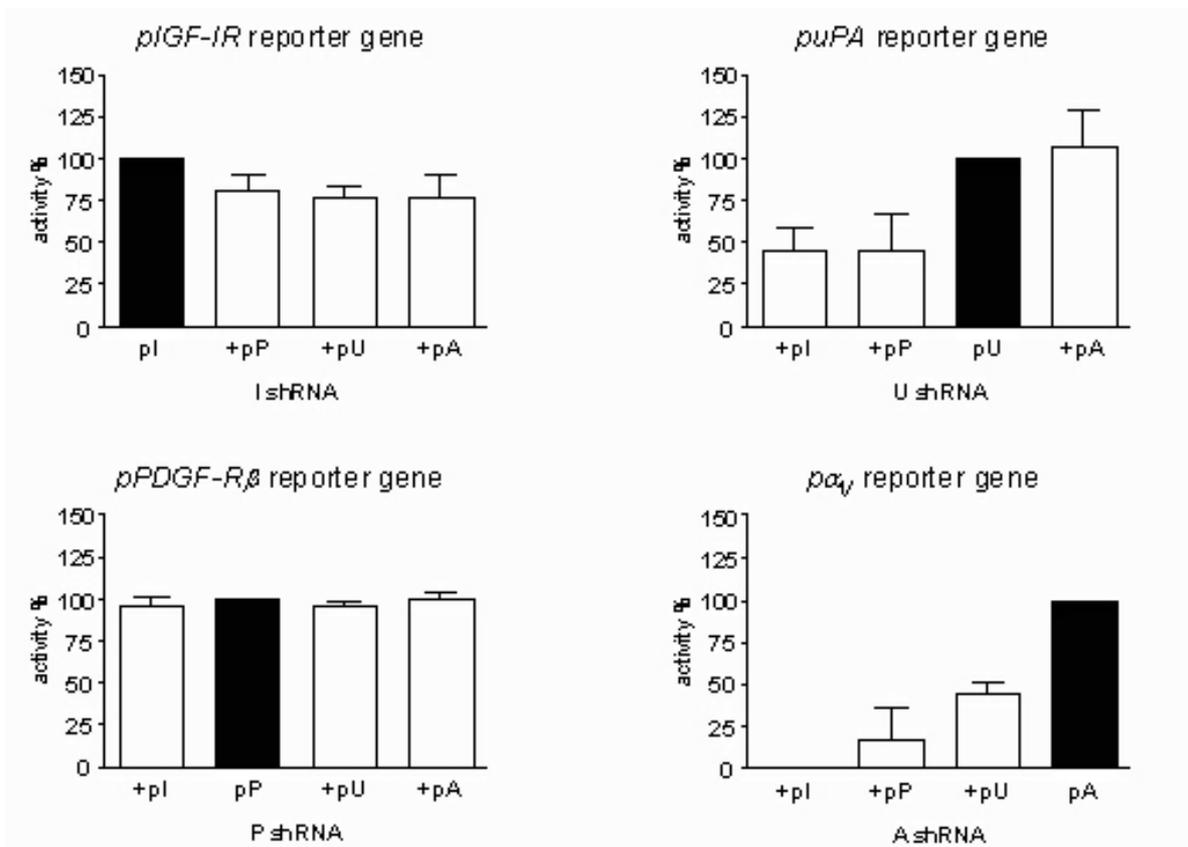


Figure 3. Effects of competition among the different shRNA-expressing plasmids. Each shRNA expressing plasmid (160 pM) was transfected into HEK293T cells with its EGFP hybrid reporter plasmid (40 pM) and with each of the other shRNA expressing plasmids (160 pM). The intensity of cellular EGFP fluorescence was measured at 36 h after transfection. The results are expressed in percentage relative to the fluorescence of cells transfected with the each of the reporter-effector pair together with empty pMCSpSUPER plasmid.

other siRNA plasmids. Most notably, however, the activity of pU (*puPA* reporter gene) was reduced by 54% and that of pA (*p α_v* reporter gene) was virtually abolished by the simultaneous expression of pI or pP. Taken all the combinations of **Figure 3**, the results clearly indicate that “stronger” siRNAs (pP and pI) work at the detriment of “weaker” ones (pU and pA).

C. Construction and validation of bispecific shRNAs vector

As the order of KD values correlates with the hierarchy established by the competition experiments, we assembled pP and pI with the expectation that transcripts of the bi-specific pIP plasmid should be still active in achieving optimal gene knock-down of target mRNA. Hence, we started out by adapting the cassette multimerization procedure of Robinett (Robinett et al, 1996) to the construction of the bi-specific plasmid pIP (**Figure 5A**), which derives from the assembly of pI and pP. The procedure is described in the legend of **Figure 4**. To demonstrate that the bi-specific plasmid is able to simultaneously deliver transcriptionally active shRNAs genes, we measured the expression of shRNAs and their efficiency to knock-down the reporter gene when expressed from both the single-copy and the multi-copy plasmid. We observed that the P shRNA and the I shRNA were transcribed from the 1-copy and the 2-copy plasmids at comparable level (**Figure 5B**) and that transcripts from

the 2-copy plasmid were as effective as those from the one-copy plasmids to knock-down EGFP expression (**Figure 5C**).

IV. Discussion

Short interfering RNAs are successfully employed to knock-down gene expression for both functional genomics and therapeutic purposes. The possibility to construct vectors for the expression of siRNAs targeted against more than one gene would be a further challenge in RNAi applications. All the isoforms of the same gene or a cluster of genes involved in the same pathway could be knocked-down at once, with a conceivable increase in efficiency, especially in the study of complex signal transduction cascades and in the therapy of multifactorial diseases. This implies that to construct a multi-shRNA expression plasmid, the knowledge whether the selected siRNAs compete or not to each other is crucial.

In this work, we considered four genes the simultaneous downregulation of which would be beneficial for the prevention of restenosis. Using the knock-down of the EGFP reporter gene, we determined dose response curves of shRNA expressing plasmids against their own target genes. From each curve we derived the maximal activity and KD. By ordering activity values (P>A>I>U) and KD values (P>I>U>A), it came out that the two ranks are not coincident, indicating that KD accounts for some other characteristic of siRNAs.

Interestingly, the rank established by the competition experiments ($P > I > U > A$) well correlated with that of KD values ($P > I > U > A$), so that stronger shRNAs were those with lower KD ($pP = 1.73\text{pM}$ and $pI = 2.19\text{pM}$), whereas weaker siRNAs were those with higher KD ($pU = 2.40\text{pM}$ and $pA = 6.11\text{pM}$). All together these findings indicate that shRNA with very close KD values can be used for the

simultaneous expression inside cells. Indeed, when we assembled pP and pI to construct the bi-specific plasmid pPI, it resulted that both cassettes were transcribed and that the transcripts were comparably active. This indicates the feasibility of the multi-copy shRNA cassette delivery approach and its efficacy in achieving optimal knockdown efficiency.

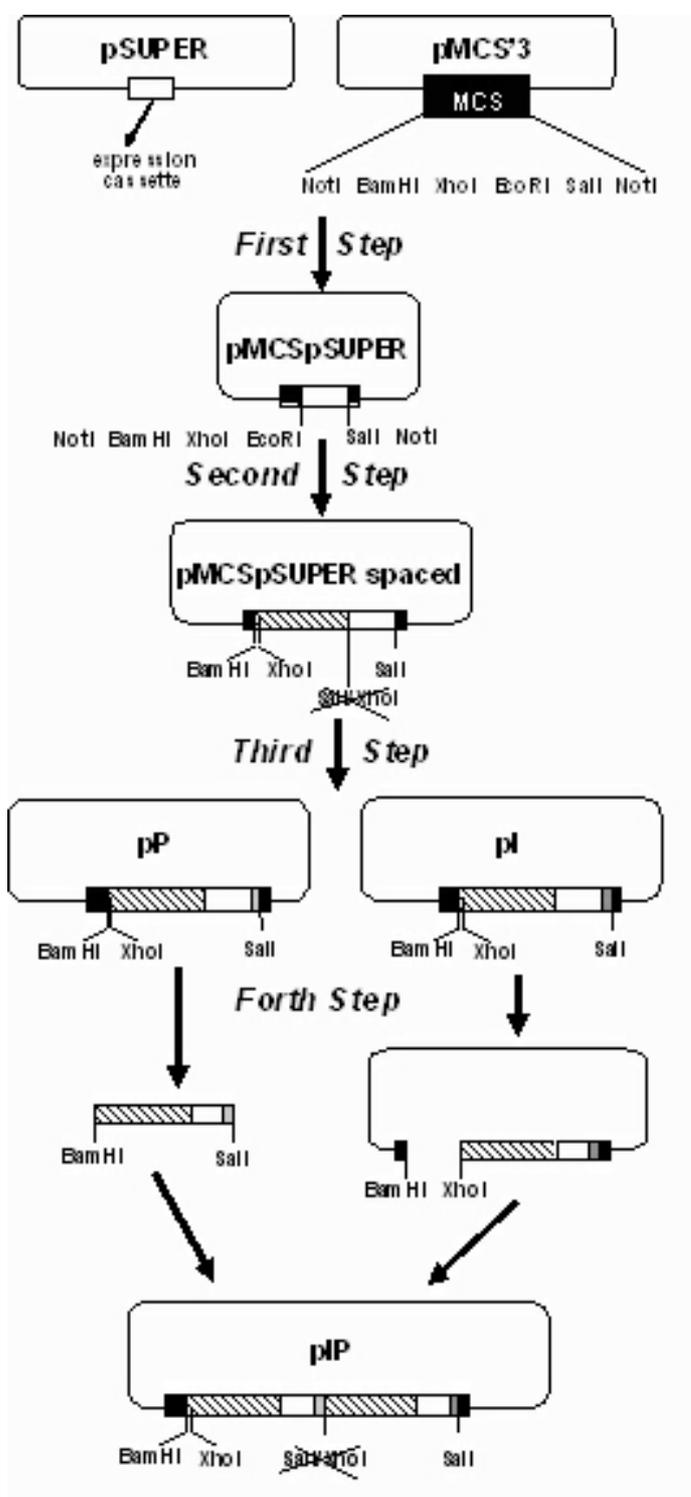


Figure 4. Construction of a multi-shRNA expressing plasmid.

Schematic representation of the strategy used to construct the bi-specific pIP plasmid. *First Step:* Cloning of the pSUPER expression cassette (Brummelkamp et al, 2002) into the pMCS'3 plasmid using Eco RI and Sal I restriction sites, to obtain pMCSpSUPER plasmid. pMCS'3 contains a multiple cloning site (MCS) flanked by Not I restriction sites in the pCMV-MCS backbone (Stratagene). *Second Step:* Cloning of the 600 bp spacer. An irrelevant 600 bp DNA PCR product, containing restriction sites for Sal I at one end and for Xho I and Bam HI at the other end, was cloned into pMCSpSUPER using the Sal I and Bam HI restriction sites. Sal I and Xho I restriction products are compatible for ligation and reconstitute a site that cannot be re-cut by either enzyme. As a consequence, both Sal I and Xho I are maintained as unique sites within the spaced pMCSpSUPER plasmid. *Third Step:* Cloning of the hairpin I siRNA and P siRNA sequence into the pSUPER cassette of spaced pMCSpSUPER, to obtain pI and pP, respectively. *Fourth Step:* The pP cassette was extracted using the Sal I and Bam HI restriction sites and cloned into the pI plasmid by using the Xho I and Bam HI restriction sites. The fourth step can be repeated, each time leading to the duplication of the number of the shRNA cassettes contained within the pMCS'3 plasmid. For all cloning steps, the recA- *E. coli* strain Stb12 was used. By using the Not I restriction sites in the MCS, an insert containing all the shRNA cassettes can be easily extracted and cloned into any other suitable vector for *in vivo* transduction. White box: 300 bp pSUPER expression cassette; dark gray box: hairpin I siRNA sequence; light gray box: hairpin P siRNA sequence; dashed box: 600 bp spacer.

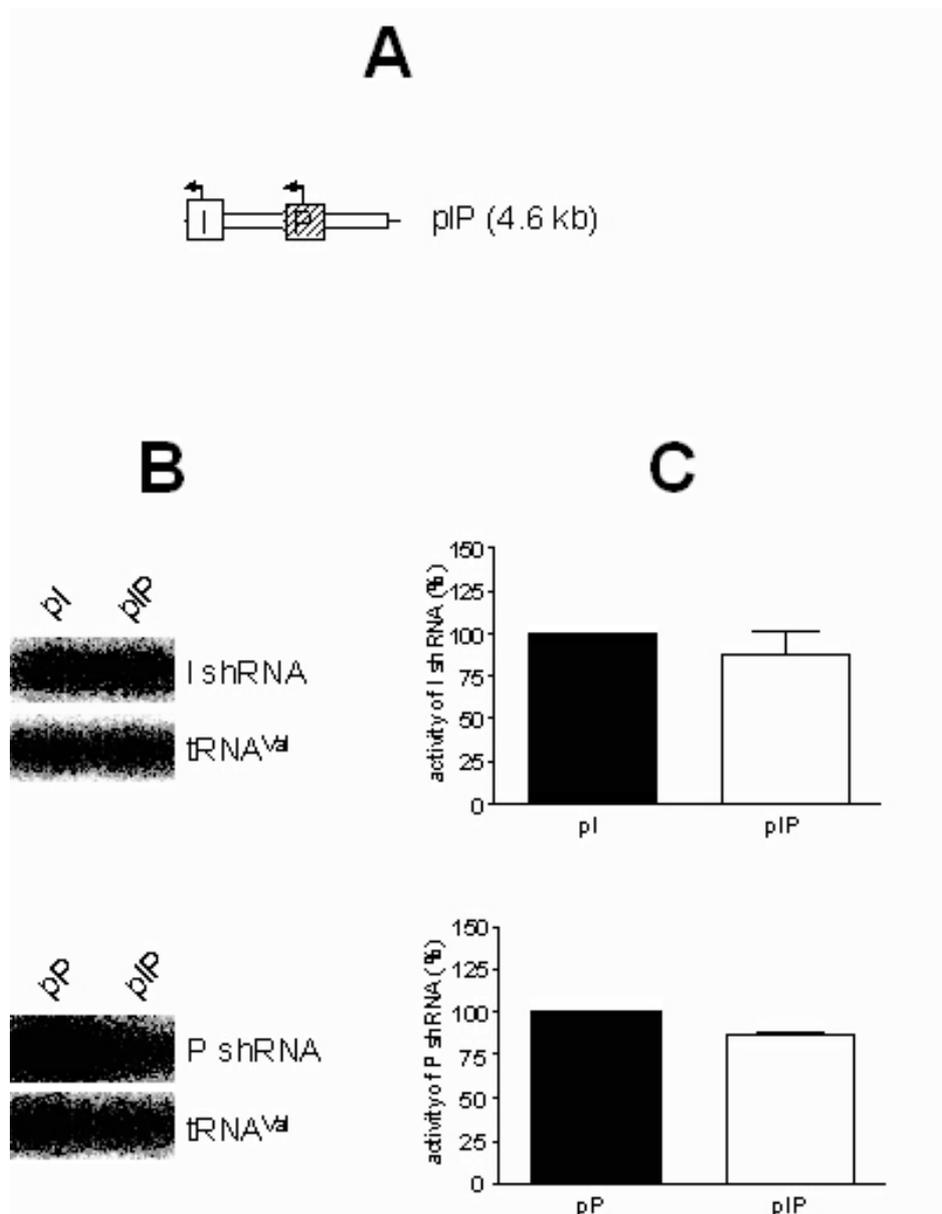


Figure 5. Multi-specific multi-shRNA plasmid transcription and activity. (A) Schematic representation of the bi-specific pIP plasmid. Arrows indicate the direction of transcription. (B) Northern blot of hairpin I and P siRNA transcripts. HEK293T cells were transfected with 450 pM of the mono-specific pI and pP and the bi-specific pIP plasmid. After three days, total RNA was extracted and analyzed by northern blotting. A representative experiment of three is reported. (C) 40pM hybrid *pIGF-IR* (upper part) or *pPDGF-R β* (lower part) reporter plasmid were cotransfected into HEK293T cells with their respective mono-specific shRNA plasmid or with the bi-specific pIP shRNA plasmid (160 pM). Cellular fluorescence was evaluated 36 h after transfection. The values are the mean \pm SD of three independent experiments and represent the reduction in fluorescence expressed in percentage relative to the cells transfected with the mono-specific shRNA expressing plasmid.

In conclusion, this work demonstrates that KD accounts for the competition between siRNAs and that expression cassettes for different shRNAs can be efficiently and rapidly assembled into a multi-specific multi-shRNA plasmids. A practical correlate of these observations is that, in order to obtain effective multi-gene knock-down, only siRNAs with similar inhibitory kinetic need to be delivered into cells. The goal of selecting such siRNAs can be easily met by the systematic analysis of their dose-response curves.

References

- Anderson J, Banerjee A, Akkina R (2003) Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4, and CCR5 confer HIV-1 resistance. *Oligonucleotides* 13, 303-312.
- Brummelkamp TR, Bernards R, Agami R (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. *Cancer Cell* 2, 243-247.
- Czauderna F, Fechtner M, Aygun H, Arnold W, Klippel A, Giese K, Kaufmann J (2003) Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA. *Nucleic Acids Res* 31, 670-682.

- Elbashir SM, Harborth J, Weber K, Tuschl T (2002) Analysis of gene function in somatic mammalian cells using small interfering RNAs. **Methods** 26, 199-213.
- Holen T, Amarzguioui M, Wiiger MT, Babaie E, Prydz H (2002) Positional effects of short interfering RNAs targeting the human coagulation trigger Tissue Factor. **Nucleic Acids Res** 30, 1757-1766.
- Hsieh AC, Bo R, Manola J, Vazquez F, Bare O, Khvorova A, Scaringe S, Sellers WR (2004) A library of siRNA duplexes targeting the phosphoinositide 3-kinase pathway: determinants of gene silencing for use in cell-based screens. **Nucleic Acids Res** 32, 893-901.
- Kawasaki H, Suyama E, Iyo M, Taira K (2003) siRNAs generated by recombinant human Dicer induce specific and significant but target site-independent gene silencing in human cells. **Nucleic Acids Res** 31, 981-987.
- Khvorova A, Reynolds A, Jayasena SD (2003) Functional siRNAs and miRNAs exhibit strand bias. **Cell** 115, 209-216.
- Kopp CW, de Martin R (2004) Gene therapy approaches for the prevention of restenosis. **Curr Vasc Pharmacol** 2, 183-189.
- Leirdal M, Sioud M (2002) Gene silencing in mammalian cells by preformed small RNA duplexes. **Biochem Biophys Res Commun** 295, 744-748.
- McManus MT, Haines BB, Dillon CP, Whitehurst CE, van Parijs L, Chen J, Sharp PA (2002) Small interfering RNA-mediated gene silencing in T lymphocytes. **J Immunol** 169, 5754-5760.
- Poliseno L, Evangelista M, Mercatanti A, Mariani L, Citti L, Rainaldi G (2004) The energy profiling of short interfering RNAs is highly predictive of their activity. **Oligonucleotides** 14, 227-232.
- Robinett CC, Straight A, Li G, Willhelm C, Sudlow G, Murray A, Belmont AS (1996) In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. **J Cell Biol** 135, 1685-1700.
- Schuck S, Manninen A, Honsho M, Fullekrug J, Simons K (2004) Generation of single and double knockdowns in polarized epithelial cells by retrovirus-mediated RNA interference. **Proc Natl Acad Sci U S A** 101, 4912-4917.
- Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD (2003) Asymmetry in the assembly of the RNAi enzyme complex. **Cell** 115, 199-208.
- Wunsche W, Sczakiel G (2005) The activity of siRNA in mammalian cells is related to the kinetics of siRNA-target recognition *in vitro*: mechanistic implications. **J Mol Biol** 345, 203-209.
- Yu JY, Taylor J, DeRuiter SL, Vojtek AB, Turner DL (2003) Simultaneous inhibition of GSK3 α and GSK3 β using hairpin siRNA expression vectors. **Mol Ther** 7, 228-236.

