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Silencing of Bruton's tyrosine kinase (Btk) using short interfering RNA duplexes (siRNA)

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Abstract **Tec family tyrosine kinases, Bruton's tyrosine kinase (Btk), Itk, Bmx, Tec, and Txk, are multi-domain proteins involved in hematopoietic signaling. Here, we demonstrate that human Btk protein can transiently be depleted using double-stranded short RNA interference (siRNA) oligonucleotides. Imaging and Western blotting analysis demonstrate that Btk expression is down regulated in heterologous systems as well as in hematopoietic lineages, following transfection or micro-injection of Btk siRNA duplexes. The induction of histamine release, a pro-inflammatory mediator, in RBL-2H3 mast cells was reduced by 20–25% upon Btk down regulation. Similar, results were obtained when the Btk activity was inhibited using the kinase blocker LFM-A13. These results demonstrate a direct role of Btk for the efficient secretion of histamine in allergic responses. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Immunodeficiency; X-linked agammaglobulinemia; Mast cell; Allergy; Btk; Tec; Bmx; siRNA; RNAi

1. Introduction

Protein tyrosine kinases (PTKs) of the Tec family, Bruton's tyrosine kinase (Btk), Itk, Bmx, Tec and Txk, are expressed differentially in hematopoietic lineages and play a central role in signaling through immunoreceptors, such as T-cell receptor, B-cell receptor (BCR) and Fcε receptor. In B-cells, Btk is a central player in cytoplasmic B-cell signaling initiated by the antigen receptor (BCR) [1]. Upon antigen engagement, the receptor complexes, which contain multiple immunoreceptor tyrosine-based activation motifs, are coupled to a cascade of tyrosine phosphorylations initiated by cytosolic PTKs, which belong to Src, Syk, Jak and Tec families (for recent review see [1,2]). The importance of Tec family kinases was immediately highlighted by the discovery of mutations in Btk in humans diagnosed with X-linked agammaglobulinemia and in mice with X-linked immunodeficiency (Xid) [3–6]. Furthermore, tyrosine phosphorylations and membrane translocation of Btk take place upon activation of mast cells by antigen/IgE-mediated cross-linking of the FcεRI receptor [7–10]. In mast cells, Btk mutations lead to impairment in pro-inflammatory cytokine production [11], intracellular calcium influx and granule exocytosis [12], induced by cross-linking of high-affinity IgE receptor on mast cells. Simultaneously, a vast number of signaling molecules have been reported to be involved and take a part in the signal transduction of Tec family kinases (for an overview see [2,13,14]). Post-transcriptional gene silencing by double-stranded RNA interference (RNAi) was first observed from studies in *Caenorhabditis elegans* and *Drosophila melanogaster* and was subsequently found in other organisms (for review see [15,16]). The use of RNAi in mammalian studies has only recently been established by introducing short interference (21 bp) duplex RNA (siRNA) [17,18].

In this study, we use the siRNA strategy for blocking Btk expression in hematopoietic cells and to examine consequences of the loss-of-function. We have designed three different siRNA duplexes targeted to different positions in the *BTK* gene. We were able to block Btk expression in a variety of cell types including human preB-cells and B-cells, mouse B-cells and in rat RBL-2H3 mast cells.

2. Materials and methods

2.1. Construction of siRNA duplexes

The siRNA duplexes used in our study are designed according to the guidelines described earlier [17–19]. For Btk and Bmx, the sequences were chosen to target identical sequences for both human and mouse genes. The alignment procedure was as described earlier [13]. All siRNA duplexes were synthesized with 2 nt deoxythymidine 3′-overhangs (Xeragon, USA) as described by Elbashir et al. [19]. The cDNA-targeted region and the sequence of the siRNA duplexes, for Btk, Bmx and enhanced green fluorescent protein (EGFP), are as follows: Btk siRNA-1 (accession no. NM_000061 and L29788); targeted region (cDNA): ^{1682}-TTGGTAAACGATCAAGGAG-^{1700}; sense siRNA: 5′-UUGGUAAACGAUCAAGGAGUU; antisense siRNA: UUAACCAUUUGCUAGUUCCUC-5′; Btk siRNA-2; targeted region (cDNA): ^{895}-GGGAAAGAAGGAGGTTTCA-^{913}; sense siRNA: 5′-GGGAAAGAAGGAGGUUUCAUU; antisense siRNA: UUCCCUUUCUUCCUCCAAAGU-5′; Btk siRNA-3; targeted region (cDNA): ^{518}-GAAGCTTAAAACCTGGGAG-^{536}; sense siRNA: 5′-GAAGCUUAAAACCUGGGAGUU; antisense siRNA: UUCUUCGAAUUUUCCACCCUC-5′; Bmx siRNA (accession no. NM_001721 and NM_009759); targeted region (cDNA): ^{943}-AAAGAAGGAGCATTTATGG-^{961}; sense siRNA: 5′-AAAGAAGGAGCAUUUAUGGUU; antisense siRNA: UUUUUCUUCCUCGUAAAUACC-5′; EGFP siRNA (accession no. AF435433, as described in [20]); targeted region (cDNA): ^{28}-GGAGTTGTCCCAATTCTTG-^{46}; sense siRNA: 5′-GGAGUUGUCCCAAUUCUUGUU; antisense siRNA: UUCCUCAACAGGGUUAAGAAC-5′.

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Abbreviations: Btk, Bruton's tyrosine kinase; FcεRI, high-affinity IgE receptor of mast cells; LFM-A13, α-cyano-β-hydroxy-β-methyl-*N*-(2,5-dibromophenyl) propenamide; EGFP, enhanced green fluorescent protein; BCR, B-cell receptor

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2.2. Transfections

HEK-293T, NIH-3T3, Cos-7 and rat mast cells RBL-2H3 were cultured in Dulbecco's modified Eagle's medium (Gibco/BRL) and WEHI-231, NALM-6, Ramos and Raji cells were cultured in RPMI 1640 (Gibco/BRL) as described earlier [10]. Hematopoietic cells were transfected with siRNA using Oligofectamine (Invitrogen) in Opti-MEM® 1 medium (Gibco/BRL) according to the manufacturer's instructions. FuGENE6 (Roche) was used for transfection of HEK-293T and NIH-3T3 cells with the Btk-EGFP plasmid. The Btk-EGFP plasmid was described earlier [10]. After 48 h incubation cells were fixed as described [21] and stained with an affinity-purified anti-Btk-SH3 polyclonal antibody [10] visualized with FITC-conjugated donkey anti-rabbit IgG (Jackson Laboratories Inc.).

2.3. Immunofluorescence and microinjections

NIH-3T3 cells were grown to 50% confluency on 22 mm cover-slips and were co-injected with 100 nM triplet siRNA duplexes and 100 ng/μl Btk-EGFP plasmid into the cytoplasm using an Eppendorf 5246 Microinjector. For injection marker, 1 μg/μl dextran-tetramethylrhodamine 70 kDa (Molecular Probes) was used. After 24 h incubation cells were fixed, stained and visualized as described earlier. [10].

2.4. Biochemical analysis of gene expression

Cellular protein lysates were resolved on SDS–PAGE and then transferred to nitrocellulose membrane as described earlier [22]. The protein expression was analyzed using monoclonal anti-Btk (Becton Dickinson PharMingen) and monoclonal anti-β-actin (Sigma).

2.5. Histamine release assay

The mast cell line, RBL-2H3, was transfected with 100 nM triplet siRNA duplexes. After 48 h cells were sensitized by anti-DNP IgE (500 ng/ml for 12 h) and stimulated with 1 μg/ml DNP–bovine serum albumin (Sigma). The reaction was stopped on ice after 15 min induction. The histamine release was determined using enzyme-linked immunosorbent assay (ELISA) (IBL GmbH, Germany). For drug-induced inhibition, RBL-2H3 cells were incubated with 100 μM Btk inhibitor, LFM-A13 (α-cyano-β-hydroxy-β-methyl-*N*-(2,5-dibromophenyl) propenamide) [23], 30 min prior to stimulation.

3. Results and discussion

3.1. Suppression of endogenous Btk by siRNA duplexes

We synthesized three different siRNA duplexes specific for Btk mRNA, the sites being conserved on both the human and the mouse gene. In addition to Btk siRNA, we made siRNA for EGFP as a control. In some experiments siRNA specific for Bmx, a non-expressed member of Tec family kinases in B-cell and mast cell lineages, was used as an additional control. Initial experiments were performed in the rat mast cell line RBL-2H3 (Fig. 1A). We observed that the Btk silencing was moderate when each of these duplexes was applied separately. However, when we combined all three siRNA duplexes

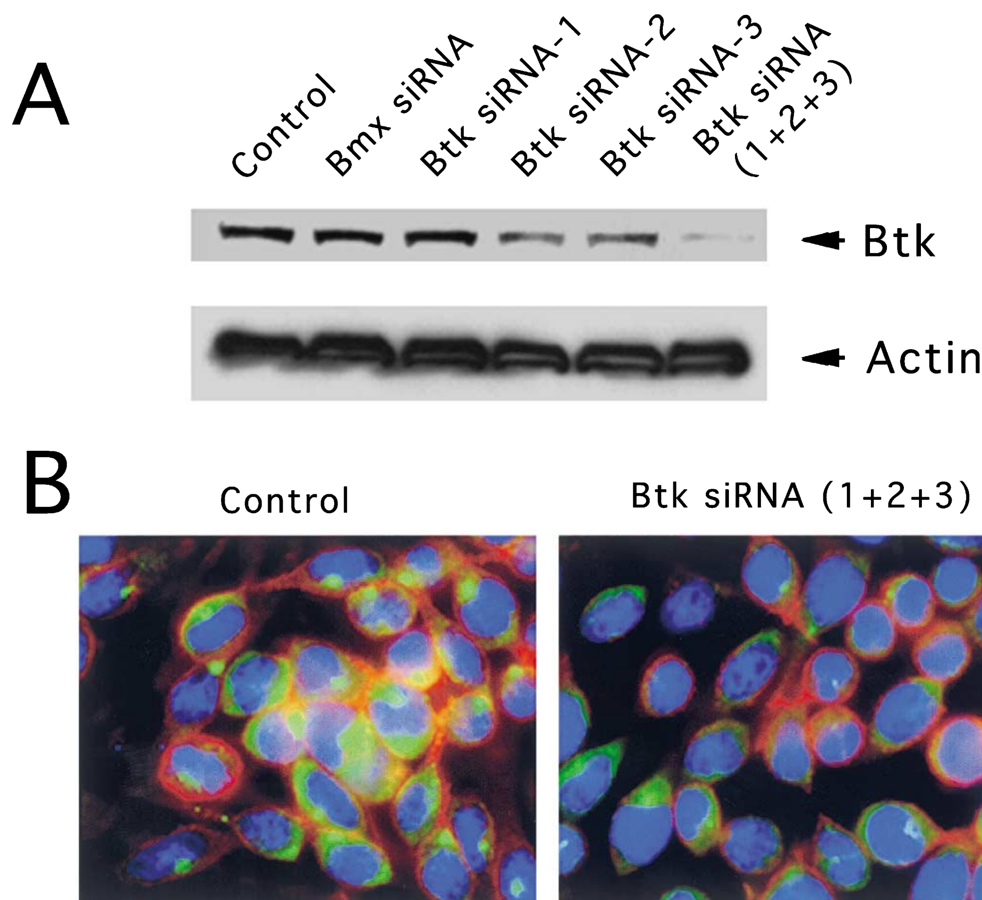

Fig. 1. Btk siRNA-mediated inhibition of endogenous Btk expression. A: Effect of Btk siRNA on endogenous Btk expression, targeted toward three different sites (1–3) in the Btk coding region. Upper: the expression of Btk in RBL-2H3 cells was analyzed 48 h post-transfection of siRNA duplexes 1–3 added separately or in triplet combination (final concentration 100 nM). For control, expression of Btk in non-transfected cells and in Bmx siRNA-transfected cells is visualized (lanes 1–2). Lower: the Btk expression was normalized to β-actin levels of total cell extracts. Western blot analysis of Btk and β-actin was performed as described in Section 2. B: Confocal images of endogenous Btk expression in RBL-2H3 mast cells 48 h after transfection with triplet Btk siRNA duplexes. Cells were fixed and stained for Btk (FITC, green), nuclear DNA (DAPI, blue) and cytoskeleton (probed with Alexa Fluor® 647 Phalloidin, Cy5, red) as described in Section 2.

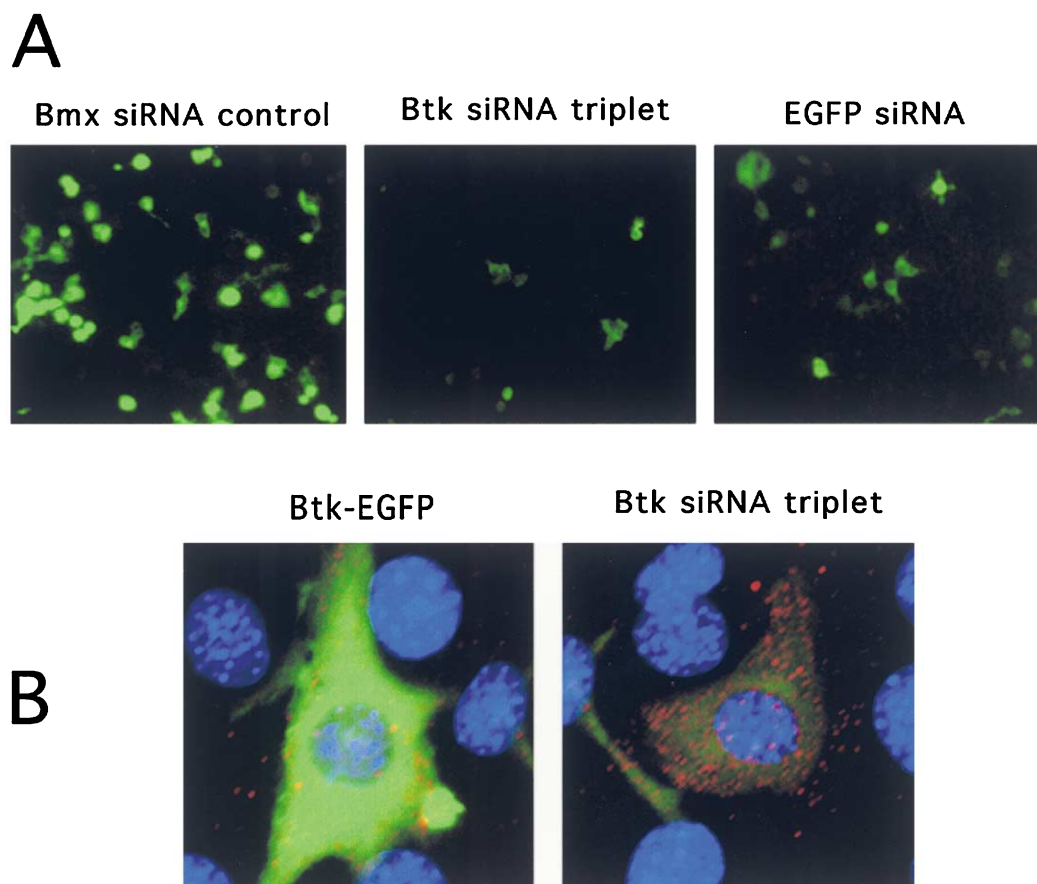

Fig. 2. Silencing of Btk-EGFP fusion gene by Btk siRNA and EGFP siRNA duplexes. A: Confocal images of Btk-EGFP expression in transiently transfected HEK-293T cells together with Bmx siRNA (non-specific siRNA control), triplet Btk siRNA and EGFP siRNA. B: Confocal images of Btk-EGFP expression in representative living NIH-3T3 cells following co-injection of Btk-EGFP plasmid, without and with triplet Btk siRNA duplexes (final concentration 100 nM). Dextran-tetramethylrhodamine 70 000 was used in order to identify injected cells. Cells were photographed at identical exposure time by confocal microscopy at 48 h post-transfection as described in Section 2.

(hereafter referred to as triplet duplexes), the Btk silencing was great, up to 90% (Fig. 1A), while Bmx siRNA (control) did not have any effect on the Btk expression pattern. Consistent with the Western blot analysis (Fig. 1A), an impaired endogenous Btk expression was observed with confocal microscopy images of RBL-2H3 cells following siRNA transfection (Fig. 1B). In agreement with Btk-EGFP localization [10], when the affinity-purified antiserum was used on Btk-EGFP-transfected Cos-7 cells a mainly cytoplasmic distribution was observed (data not shown). However, in the RBL-2H3 cells a distinct punctuated staining pattern was also seen (Fig. 1B). This perinuclear staining has been previously observed in different hematopoietic cells expressing endogenous Btk (unpublished data). The origin of this subcellular pattern is not known and the possibility existed that the antiserum cross-reacted with a protein expressed in hematopoietic cells. If this is the case, the siRNA silencing technology allowed us to re-examine and validate previous Btk stainings. Indeed, the cytoplasmic stainings and the dotted regions were similarly suppressed upon treatment of the triplet Btk siRNA duplexes (Fig. 1B). Together these data show the specificity of siRNA silencing of endogenous Btk expression and how this specific inhibition can be used to address biological questions. Further studies will be needed in order to characterize the nature of the perinuclear dot-like distribution of Btk in hematopoietic cells.

3.2. Btk silencing in heterologous cell systems

In order to characterize siRNA silencing further, we transiently transfected the Btk-EGFP plasmid into HEK-293T cells together with triplet Btk siRNA duplexes (Fig. 2A). Using the Btk specific siRNA, we were able to detect the Btk-GFP silencing in living cells, but not with the Bmx siRNA duplexes (Fig. 2A). As shown in Fig. 2A, the expression of Btk-EGFP was inhibited efficiently by Btk and as well as by

Table 1
Effect of siRNA duplexes on histamine release of IgE/antigen-stimulated rat RBL-2H3 mast cells

RBL-2H3 cells	Histamine secretion[a] (% of control)
Control (antigen/IgE-stimulated)	100[b]
+Triplet Btk siRNA duplexes	77 ± 5.1
Control (antigen/IgE-stimulated)	100
+LFM-A13	76 ± 4.2

Effect of Btk siRNA-transfected cells or treated with Btk inhibitor LFM-A13. The cells were cultured in six-well culture dishes (2 ml medium), were transfected with 100 nM Bmx siRNA (control) or 100 nM triplet Btk siRNA duplexes or incubated with 100 μM LFM-A13. Histamine secretions were analyzed by ELISA as described in Section 2.
[a]Results are averages of at least three independent experiments, each in duplicate.
[b]Absolute concentrations of histamine secretion in control experiments were 14.3 ng/ml medium.

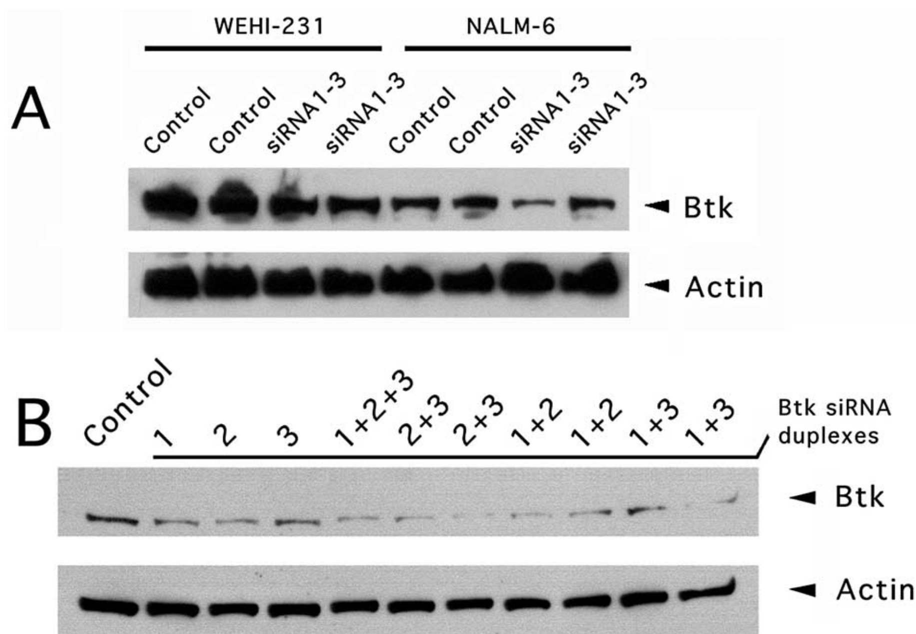

Fig. 3. Silencing of endogenous Btk in siRNA-transfected lymphocytes. A: Direct transfection of 100 nM triplet Btk siRNA duplexes into suspension of mouse B-cells (WEHI-231) and human preB-cells (NALM-6). B: Synergistic suppression of Btk in RBL-2H3 cells using Btk siRNA (1–3) duplexes. The final concentration of Btk siRNA duplexes used for transfections was kept at 100 nM. In control lanes, either EGFP siRNA or Bmx siRNA duplexes were used.

EGFP siRNA duplex. Moreover, we found no difference in the inhibition intensity of Btk-EGFP expression when we perform these experiments in Cos-7 cells (data not shown). In another experiment, we co-injected the Btk-EGFP plasmid with Btk triplet siRNA duplexes into NIH-3T3 cells (Fig. 2B), so as to follow the expression at the single cell level. In this case, we found the silencing to be essentially complete in the majority of cells.

3.3. Uptake and persistence of siRNA in hematopoietic cell lines

As shown in earlier experiments the transfection of Btk siRNA duplexes to adherent cells was very effective, since Btk expression was strongly down regulated. Therefore, we investigated the ability of hematopoietic suspension cells to take up siRNA duplexes using transient transfection protocols similar to adherent cells. Indeed, hematopoietic cells were able to take up siRNA duplexes using oligofectamine, allowing us to study suppression of genes targeted by siRNA (Fig. 3A). As indicated in Fig. 3A, the Btk suppression was lower and less efficient as compared to RBL cells, about 60–70% using the Btk triplet siRNA duplexes in mouse B-cell lymphoma WEHI-231 and human preB-cell NALM-6. Similar results were observed with human Ramos and Raji B-cell lines (data not shown). These results illustrate the use of siRNA in order to silence endogenous genes in hematopoietic cells, which generally are not easily transfectable, although transfections efficiency needs further improvement.

3.4. Synergistic effects of Btk siRNA duplexes

In the present study, the Btk siRNA duplexes synthesized against three conserved sites were shown to have differential silencing activity (Fig. 1A). As shown above, combining all three Btk siRNA duplexes induced the highest suppression. Therefore, we sought to examine the efficiency of these siRNA duplexes in combination of two (Fig. 3B). When the siRNA-1 was combined with siRNA-2 or siRNA-3, we observed a synergistic effect (Fig. 3B). In a similar manner, we observed that the combination of siRNA-2 and siRNA-3 induced more silencing than each one separately. Thus, our data indicate that when the relatively inactive siRNA-1 is combined with moderately active siRNA-2 or siRNA-3, the silencing effect was enhanced, possibly due to an altered accessibility to the mRNA secondary structure. However, as indicated in a recent work the siRNA efficiency could not be predicted prior to examination [24].

3.5. Btk silencing and allergic responses in mast cells

To study the biological relevance of endogenous Btk suppression by siRNA duplexes, we set out to investigate signaling responses of RBL-2H3 mast cells. In FcεRI-stimulated cells, the histamine release was decreased by 20–25% when Btk expression was down regulated by triplet Btk siRNA duplexes (Table 1). To validate the specificity and accuracy of our data, we compared siRNA down regulation to the Btk kinase inhibitor LFM-A13 [23]. In the presence of LFM-A13, we observed a similar level of inhibition in histamine secretion, about 20–25% (Table 1). The results indicate that Btk kinase activity is required for efficient mast cell activation and therefore possibly influences allergic responses. These observations support previous findings measuring IP_3 production, granule exocytosis and calcium influx in wild-type and/or Xid and Btk-deficient mast cells [12]. In mice with disease gene mutations compensatory mechanisms could potentially develop over time. By using siRNA or kinase inhibitors (LFM-A13) such an influence is minimized. Despite that the siRNA transfection in hematopoietic cells is less efficient as compared to adherent cells, this work shows a simple strategy to selectively impair expression.

In order to potentiate the effect of siRNA in lymphocytes,

plasmid-based delivery [25,26] combined with a selectable marker could be applied, or alternatively, the transfection procedures for siRNA oligonucleotides could be further improved. In a collaborative effort, we are currently studying a retrovirus siRNA hairpin vector system for stable and efficient 'knock-down' of gene(s) in B-cells and T-cells. Since siRNA silencing is effective and specific while keeping the cellular proteome intact, the function of many signaling molecules could be revealed, including the possibility to simultaneously target multiple genes.

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