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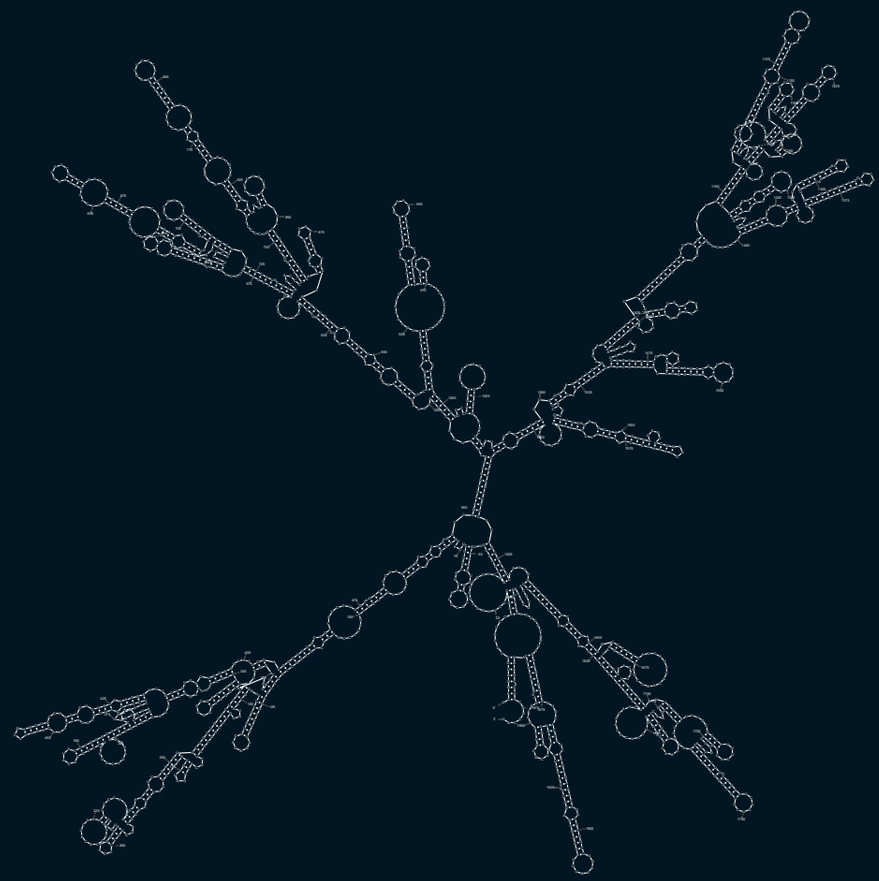

Juhana E Heinonen

Karolinska Institutet

From DEPARTMENT OF LABORATORY MEDICINE,
Clinical Research Center, Karolinska Institutet, Stockholm,
Sweden

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Juhana E. Heinonen

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to my parents

ABSTRACT

RNA mediated gene silencing, RNA interference (RNAi), has recently been identified in mammalians. This thesis describes the utilization and development of RNAi based tools for signal transduction research. The effects of Bruton's tyrosine kinase (Btk) down-regulation with RNAi have been studied with gene expression profiling in hematopoietic cells.

Btk is indispensable for the B cell development. The mutation in the *Btk* gene causes primary immunodeficiency disease X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice. Less is known about the Btk's role in other hematopoietic cells.

This work addresses Btk's role in monocytes, macrophages and mast cells. In paper I, Btk's down-regulation with short interfering RNA (siRNA) caused decreased histamine secretion in the RBL-2H3 mast cell line. Moreover, the concept of using three siRNA against the same target gene for enhanced down regulation was introduced for the first time. In paper II, U937 cell line expressing short hairpin RNA (shRNA) against Btk was created. The outcome of stable Btk down-regulation was analyzed with gene expression profiling. Btk was found to regulate 58 transcripts in macrophages (PMA stimulated U937 cells) compared to 11 in monocytes. The analysis suggests Btk's involvement in macrophage effector functions. In paper III, an inducible shRNA vector system was developed to overcome the shortcomings of constitutively expressed shRNAs. The system uses Cre recombinase-mediated site-specific recombination, H1 polymerase III promoter-driven expression and lentiviral delivery. The advantages of pLIND (LentiINDucible) vector system are, no basal shRNA expression in the inactive state, EGFP selection marker and lentiviral transduction enabling gene transfer into primary and other difficult-to-transfect cells. In paper IV, the potential Btk dependency of bone marrow-derived mast cells (BMMC) was addressed. Btk deficient BMMC were shown to grow slower compared to parental cells. This was found to be due to G2/M arrest. Moreover, it was found that Btk negatively regulates expression of novel G protein coupled receptor GPR177. This negative regulation was also found in B cells. Another affected transcript was up-regulation of melanoma antigen (Mela), which is of interest owing to Btk's role as a putative tumor suppressor gene.

LIST OF PUBLICATIONS

I. **Heinonen J.E.,** Smith C.I.E. and Nore B.F.
Silencing of Bruton's tyrosine kinase (Btk) using short interfering RNA duplexes (siRNA). (2002) *FEBS Letters* 527: 274-278.

II. **Heinonen J.E.**, Nore B.F., Lindvall J.M. and Smith C.I.E.
Stable silencing of Bruton's tyrosine kinase (Btk) in the shRNA expressing human promonocytic cell line U937. *Manuscript*

III. **Heinonen J.E.**, Mohamed A.J., Nore B.F. and Smith C.I.E.
Inducible H1 Promoter-Driven Lentiviral siRNA Expression by Stuffer Reporter Deletion. (2005) *Oligonucleotides* 15**:** 139–144.

IV. **Heinonen J.E.**, Nyström Lansner K., Blomberg K.E.M., Lindvall J.M., Mattingsdal M., Berglöf A., Grandien A. and Smith C.I.E.
Expression of novel G-protein coupled receptor 177 is negatively regulated by Bruton's tyrosine kinase (Btk) in primary bone marrow-derived mast cells. *Manuscript*

Related publications

Ge R., **Heinonen J.E.**, Svahn M.G., Mohamed A.J., Lundin K.E., Smith C.I.E. Zorro locked nucleic acid induces sequence-specific gene silencing. (2007) *FASEB J.* 21: 1902-14.

Lindvall J.M., Blomberg K.E.M, Valiaho J., Vargas L., **Heinonen J.E.**, Berglöf A., Mohamed A.J., Nore B.F., Vihinen M., Smith C.I.E.
Bruton's tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. (2005) *Immunol Rev.* 203: 200-15.

Vargas L., Nore B.F., Berglöf A., **Heinonen J.E.**, Mattsson P.T., Smith C.I.E., Mohamed A.J. Functional interaction of caveolin-1 with Bruton's tyrosine kinase and Bmx. (2002) *J Biol Chem.* 277: 9351-7.

CONTENTS

LIST OF ABBREVIATIONS

Ago	Argonaute family protein
AON	antisense oligonucleotide
AP-1	Activating Protein-1
Bcl-X_L	Bcl-2 family member long isoform
BLNK	B-cell linker
BMMC	Bone marrow-derived mast cells
CD	cluster of differentiation
DAG	Diacylglycerol
Ds	double strand
FcεRI	IgE high affinity receptor
Fc	fragment crystallizable
IBtk	inhibitor of Btk
Ig	Immunoglobulin
IL	Interleukin
IP3	inositol 1,4,5-trisphosphate
IRAK-1	interleukin-1 receptor-associated kinase 1
ITAM	immunoreceptor tyrosine-based activation motif
JNK	Jun N-terminal kinase
LPS	lipopolysaccharide
MAPK	Mitogen activated protein kinase
miRNA	Micro RNA
NF-AT	nuclear factor of activated T-cells
NF-κB	nuclear factor kappa B
Oct1	octamer-binding transcription factor-1
PBMC	peripheral blood mononuclear cells
PH	pleckstrin homology
PI3K	phosphatidylinositol 3-kinase
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLCγ2	Phospholipase C–γ2
Pol II/III	RNA polymerase II/III
PS-ODN	phosphorothioate oligodeoxynucleotide
PTK	protein tyrosine kinase
qRT-PCR	quantitative real-time PCR
RISC	RNA induced silencing complex
RNAi	RNA interference
SH	Src homology
shRNA	short hairpin RNA
siRNA	Small interfering RNA
Sp1	specificity factor-1
Ss	Single strand

STAT	signal transducer and activators of transcription
Syk	spleen tyrosine kinase
TFII-I	general transcription factor II-I
TH	Tec homology
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule 1
wt	wild type
Xid	X-linked immunodeficiency
XLA	X-linked agammaglobulinemia

1 INTRODUCTION

Bruton's tyrosine kinase (Btk) is crucial for B cell development and thereby also for the adaptive immunity. The evidence for this has been found from a disease called X-linked agammaglobulinemia (XLA) where mutation in the *BTK* gene leads to a block in B cell development. Btk is expressed in all hematopoietic cells, except T and plasma cells. The importance of Btk is not only restricted to the development of antibody producing B cells, but it has also been shown to involve innate immunity. This suggests that Btk signaling is important in other hematopoietic cells as well. The objective of this thesis is to define new signaling molecules regulated by Btk in less explored hematopoietic cells. Especially the role of Btk for monocyte, macrophage, and mast cell function is addressed. This has been approached by developing and utilizing small interfering RNA techniques belonging to a novel gene regulation process called RNA interference (RNAi). The importance and potential of RNAi to medical research was acknowledged last year, when Andrew Z. Fire and Craig C. Mello received the Nobel price in physiology and medicine for their discovery of RNAi.

1.1 Bruton's tyrosine kinase (Btk)

Btk, the first cytoplasmic protein tyrosine kinase (PTK) implicated in a hereditary disease, was identified in 1993 by two independent groups [1,2]. The *BTK* gene encodes a protein mutated in X-linked agammaglobulinemia (XLA) in humans and X-linked immunodeficiency (Xid) in mice. Mouse and human Btk share 98.3 % sequence homology with each other [3]. In humans, the Btk gene is located on the long arm of the X chromosome Xq21.3-22, composing 19 exons ranging from 55 to 560 bases and spanning a 38 kb genomic region [4,5]. The Btk promoter area, 280 bp upstream from transcription initiation site, contains three known binding sites for transcription factors. These are the GC-box and the GT-box binding transcription factors Sp1 and Sp3 and the PU-box binding transcription factors PU.1 and Spi-B [6,7]. The transcription factors Oct1 and Oct2 are able to bind in concert with the coactivator BOB.1/OBF.1 to the human and murine Btk promoters leading to their activation. Moreover, these proteins activate the murine Btk promoter synergistically with PU.1 [8].

Btk is a member of the Tec family kinases which also includes Bmx, Itk, Txk and Tec. The second largest cytoplasmic PTK family is characterized, with the exception of Txk, by the presence of a N-terminal plekstrin homology (PH) and Tec homology (TH) domain followed by three Src homology domains [9]. The N-terminal PH domain is responsible for plasma membrane localization and many characteristic interactions with signaling molecules (Fig. 1). The TH domain contains N-terminal Btk homology motif that binds to a zinc ion and the C-terminal SH3 domain binding proline-rich sequences. The SH3 domain contains a tyrosine residue at position 223, a known as the autophosphorylation site of Btk. The characteristic function of SH3 is its binding to proline rich sequences with consensus sequence PxxP [10]. The fourth Btk domain is SH2, responsible for binding phosphorylated tyrosine residues. The FLVR motif in the SH2 domain has been found essential for the recognition of phosphotyrosine motifs [11]. The largest domain in Btk is the kinase domain (SH1), that contains an ATP

binding site, a catalytic site and an activation loop containing tyrosine 551, which is the second main tyrosine phosphorylation site of Btk [12,13].

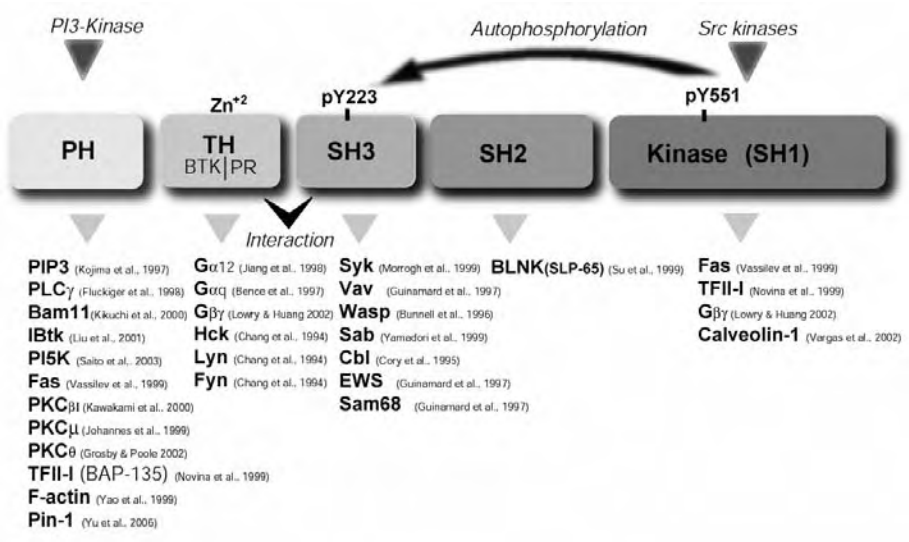


Figure 1. *The domain structure of Btk and its main known interacting partners.*

1.1.1 X-linked agammaglobulinemia (XLA) and X-linked immunodeficiency (Xid)

Mutations, spread in every part of human Btk, have been shown to cause XLA disease. XLA is an immunodeficiency disorder characterized by a lack of mature B lymphocytes which leads to notorious consequences e.g. an increased susceptibility to infections. In B cell development, XLA patients have shown to have partial block after the pro B cell stage and a complete block after the pre B cell level [14].

Xid is the XLA disease in mice caused by one specific mutation altering conserved amino acid arginine at position 28 to a cystein (R28C) [3,15]. Xid disease is a less severe immunodeficiency than XLA is in humans. Xid mice have only a partial block in the pre-B and mature B lymphocyte stages. The characteristic effect in Xid mice is decreased amounts of IgM and IgG3. Moreover, the B cells from these mice are unresponsive to the activation by mitogenic antigens as well as T-cell independent type II antigens [16,17]. Mice lacking the Btk protein (knock-out) show the same general phenotype as mice with the R28C mutation, while humans with the same mutation show a classical XLA phenotype [18-20]. This should be taken into consideration when comparing results of Btk mediated signaling between different species. In this context, our recent results revealed differences in Xid and knock-out mice transcriptomes (unpublished results). Interestingly, mice deficient in PLCγ2 [21], p85α [22], BLNK/SLP-65 [23] and PKCβ [24], the downstream molecules of B cell receptor

signaling, show a Xid-like phenotype. The fundamental role of Btk and its function in different species remain elusive.

1.1.2 Signal transduction of Btk in hematopoietic cells

Btk has been found to be involved in signaling via B cell receptor (BCR), Integrin receptor, Fc receptors, G protein-coupled receptors (GPRC), Toll-like receptors (TLR), death receptors, cytokine receptors and receptor tyrosine kinases (RTK) [25]. The signaling mediated through these receptors share sometimes common cytoplasmic targets. This is at the same time both an advantage and a disadvantage from research point of view. The fast growing number of new interaction partners provides the possibility to link Btk signaling to multiple pathways. The downside is the common problem of many bioinformatic approaches where the huge amounts of data can mask important facts.

In addition to plasma membrane localization, Btk has been characterized as having another spatio-functional property, the nucleocytoplasmic shuttling. Btk has been shown to translocate to the nucleus upon growth factor stimulation and cross-linking of the BCR. This shuttling property of Btk is not fully understood, since deletion of the PH domain leads to an even distribution of Btk between the nucleus and cytoplasm whereas deletion of an SH3 domain leads to predominantly nuclear localization of Btk [26].

1.1.3 Btk in B cells

The main known function of Btk is in the B cell development. The most studied and best known pathway for Btk activation is the BCR pathway. In addition to the BCR pathway, stimulation of various co-receptors including CD38, CD19, CD40, CD72, IL-6R and IL-5R, results in Btk activation and further signaling leading to cell proliferation and/or differentiation of B cells. These complex pathways are only partly understood and needs to be further studied. TLRs are expressed mainly on mast cells, macrophages and dendritic cells, but also on B cells [27]. Btk deficient B cells show reduced proliferation on lipopolysaccharide (LPS) stimulation [28]. This signaling is mediated through TLR4 and RP105 in mouse B cells [29,30]. Integrins are important for migration, recirculation and homing of B cells. Btk has been found to be important for BCR induced integrin $\alpha 4\beta 1$-mediated adhesion to VCAM-1/CD106 and fibronectin. These processes involve IP3 receptor and PKC mediated integrin clustering and cytoskeletal reorganization [31].

BCR

After BCR cross-linking, the tyrosines within the immunoreceptor tyrosine-based activation motif (ITAM) are phosphorylated by members of the Src family of tyrosine kinases. This allows the recruitment and activation of Syk, which in turn leads to phosphorylation of the BLNK adaptor protein. Activated Syk is also involved in tyrosine phosphorylation of other possible adapter proteins recruiting the PI3 kinase to

the membrane. Activated PI3 kinase generates PIP3, which binds to and activates Btk. Activated Btk binds to phosphorylated BLNK via the SH2 domain requiting PLCγ in the close proximity. This membrane localized complex of proteins is sometimes referred to as a signalosome. As a result, Btk activates PLCγ by phosphorylation and allows its catalyzation of PIP2 to second messengers IP3 and DAG. This is a central event that results in further activation of multiple signaling cascades, including IP3 induced calcium flux and NFAT translocation into the nucleus, DAG production and activation of ERK and PKCβ activation leading to activation of the transcription factor NF-κB (Fig. 1.).

Downstream signaling

Btk is involved in calcium release as previously described. Increased Ca^{2+} concentration will lead activation of calmodulin-activated serine/threonine phosphatase calcineurin. Calcineurin dephosphorylates NF-AT transcription factors and enables them to translocate into the nucleus where they activate gene transcription [32]. Similar to the Lyn-Syk-Btk-PLCgamma-Ca^{2+} -dependent activation mechanism of NF-AT, serum response factor (SRF) is activated following BCR stimulation [33]. Btk has been associated to the activation of ERK2 and JNK MAP kinases in the DT40 B cell line [34,35].

IgM-, but not CD40L-, stimulated B cells from Xid mice showed greatly diminished NF-κB nuclear translocation and DNA binding [32,33]. This and other studies confirmed BCR mediated Btk-dependent NF-kB activation [36]. Btk mediated NF-κB activation has been shown to transcriptionally activate the anti-apoptotic Bcl-2 family member Bcl-X_L [37]. Similarly, PKCβ has been shown to be needed for Bcl-X_L and NF-kB activity during the BCR stimulation [38]. Btk has been shown to regulate PKB/Akt activity [39]. Moreover, Btk was found to phosphorylate PKB/Akt in a H_2O_2 stimulated DT40 B cell line [35].

Btk can promote its own sustained activation through a positive feed back loop. This is mediated via its binding to Phosphatidylinositol 5-Kinase (PI5K), translocation to the membrane. In the membrane PI5K will convert $P_{(4)}IP$ to $P_{(4,5)}IP2$ thereby providing substrate to PI3K and prolonging the activation signal cascade [40].

As described in the figure 1, Btk does not have a carboxy-terminal negative regulator domain. Instead, Btk is known to be regulated by different cytoplasmic proteins. In this context, over expression of Sab has been shown to down-regulate Btk tyrosine phosporylation [41]. Moreover, inhibitor of Btk (IBtk) has been shown to bind to PH domain and also inhibit Btk kinase activity, BCR induced Ca2+ and NF-κB signaling [42]. Nevertheless of numerous trials we could not up-regulate Btk activity by down regulation of IBtk (Heinonen *et al.*, unpublished results). The relationship between IBtk and Btk needs to be further confirmed. PKCβ [43] and 5' inositol phosphatase SHIP1 [44] have shown to down-regulate Btk activity and its membrane localization. Btk has

been shown to physically interact with the peptidylprolyl *cis/trans* isomerase (PPIase) Pin-1. Moreover, Btk was found to get de-phosphorylated by the Pin-1 [45].

Btk has been related to apoptotic cell death in many cells. In DT40 and Nalm-6 B cell lines, Btk interacts with Fas/CD95 receptor via its PH and kinase domains preventing Fas-FADD interaction [46]. These cells will be resistant to Fas mediated apoptosis, suggesting Btk role in anti-apoptotic processes. Contradictory, Btk has also been found to mediate radiation-induced pro-apoptotic signaling in DT40 chicken B cell line [47] and in mast cells were wt cell death was more recurrent compare to Btk deficient cells [48].

Upon stimulation with IL-4 and IgM, B cells from Xid mouse has been shown to fail the S-phase entry [49]. As the role of Btk in apoptosis is not fully consistent, the data involving cyclin dependency is not clear either; anti-Ig stimulated Xid B cells express normal or almost normal levels of Cyclin D2 and D3 [50], whereas others showed that Xid B cells fail to induce cyclin D2, D3, A and E [49,51]. Interestingly, it has also been shown that upon stimulation with IL-4 and IgM Xid B cells decrease their proliferation capacity, up-regulate early activation markers CD69 and B7-2 and show bigger cell sizes [49]. We have seen similar behavior of mouse spleen B cells where cells from Xid were considerably larger compared to wt (Heinonen *et al.* unpublished results). Btk knock-out B cells were smaller compared wt or Xid. The discovery was confirmed in DT40 cells where the wt phenotype was rescued by addition of the human Btk into B7-10 (Btk knock-out) cells (Heinonen *et al.* unpublished results).

Transcription factors targets

Upon BCR cross linking, Btk phosphorylates BAP-135/TFII-I, thus enabling its nuclear translocation [52,53]. Furthermore, Btk has been proposed to regulate gene transcription in IL-5 stimulated B cells by activating BAM11 and the chromatin remodeling and transcription activation complex SWI/SNF via TFII-I activation [54]. However, a physiological role of BAM11 is unclear.

Btk has been shown to phosphorylate STAT5A in chicken B cells [55]. STAT5 is reported to be able to activate the cyclin D2 promoter in IL-2 stimulated T cells and IL-3 stimulated pro B cells [56]. It is therefore possible, that not only NF-kB, but also STAT5 might have a role in activating cyclin D2 transcription in response to BCR signalling via the B cell signalosome.

1.1.4 Btk in monocytes and macrophages

Monocytes and macrophages develop from bone marrow derived precursor cells. Macrophages play an important role in innate immunity as they recognize the foreign pathogen followed by phagocytosis. Btk signaling has been proposed to be involved in macrophage effector functions.

Toll-like receptors (TLRs)

TLRs are believed to play a key role in the innate immune system. Stimulation of TLR 2 and 4 induces phosphorylation of Btk in human monocytes and macrophages [57]. In this context, Btk has been shown to phosphorylate MyD88 adapter-like (Mal) [58]. In addition, Btk has been shown to associate with the protein complex consisting, MyD88, Mal (MyD88 adapter-like protein), and interleukin-1 receptor-associated kinase-1 (IRAK-1), but not TRAF-6 [59]. Activation of this complex leads to NF-κB activation. Stimulation with multiple ligands of TLR receptors in Btk deficient bone marrow-derived macrophages (BMDM) results in decreased amount of IL-10 secretion compared to wt [60]. This phenotype correlates with Btk-dependent induction of NF-κB and AP-1 DNA binding activity. It has been suggested that IL-10 secretion might result in decreased IL-6 secretion. As mentioned earlier human and mouse cells do not contain uniform signaling machineries, which adds an additional degree of complexity more. Related to this, studies from primary human peripheral blood monocytes (PBMC) have shown that in TLR-2 and 4 signaling Btk is needed for TNF-α and IL-1β secretion but not IL-6, IL-8 and IL-10 [61]. Whether this discrepancy with earlier described mechanism is due to the fundamental differences in mouse and human is not clear. Macrophages from Xid mice have been reported to have decreased nitric oxide production which could result in a defect in the innate immune response to bacteria and elevated IL-12 expression [62]. In fact, Btk has been shown to be involved in many macrophage effector functions, including bactericidal activity and secretion of proinflammatory cytokines (TNF-α, IL-1α) [63].

In spite of the fact that most of the previous studies have been done in mice, monocytes and macrophages from XLA patients are also suggestive of defects in effector functions. These cells have impaired capacity for chemotaxis and phagocytosis suggesting defects of the innate immune system in XLA patients [64]. Some of these defects could be caused by defects in integrin signaling. Fibronectin will activate β1 integrins, which leads to NF-κB activation in a PI3K-dependent, ERK-independent manner in monocytes [65-67]. However, recently published work reveals that Btk is not essential for LPS induced TNF-α and IL-6 production from human monocytes. Moreover, Erk1/Erk2, JNK, and p38 phosphorylation are not affected by the complete absence of Btk in these monocytes [68]. To conclude, the majority of studies suggest minor role for Btk in cytokine secretion in LPS induced monocyte signaling.

Proposed Btk mediated signaling

This discrepancy between XLA in humans and Xid in mice is not dependent on the R28C mutation, since the Btk knock-out mice behave similarly at least from the cytokine secretion point of view. The difference between human and mouse cell signalling is a challenge that the scientific community needs to take in to consideration. One potent approach has been used in Paper II, where RNA interference (RNAi) technology was utilized to create a human knock down cell line complementary to knock-out cells from mice. In this work evidence was found that support the importance of Btk for the integrin mediated macrophage effector functions. The Btk

knock down was found to decrease Integrin β1 expression in PMA stimulated U937 cells.

Btk is phosphorylated in PMA pre-treated U937 cells followed by FcαR cross-linking [69]. The fact that Btk phosphorylation was only detected after prolonged treatment with PMA supports the fact that Btk is more important to monocyte differentiation towards macrophages than to monocyte signaling. FcαR has been shown to localize in lipid rafts after receptor cross-linking [70]. We have previously shown that Btk will functionally interact with Calveolin-1, a major structural component of lipid rafts [71]. Moreover, FcαR cross-linking also causes sustained association of Btk, B-lymphocyte kinase (Blk), Syk and PKCα to rafts, whereas PI 3-kinase, PDK1 and PKCε have a transient association with rafts [70]. This is consistent with temporally regulated divergence of FcαR signaling pathways in rafts [70,72]. In paper II we show that FCAR was up-regulated ~2-fold in Btk knock down PMA stimulated U937 cells.

1.1.5 Btk in mast cells

Mast cells are generated by different precursor cells in the bone marrow. They are best known for their potent effector functions in allergic disorders, but play also an important role in host defense against bacterial pathogens. Mast cell functions can be divided to two major categories: 1) Immediate release of preformed mediators such as, histamine, proteoglycans, neutral proteases and certain cytokines for example TNF, and 2) Production of several proinflammatory, anti-inflammatory, immunoregulatory mediators such as new cytokines, chemokines, lipid-mediators followed by mast cell activation [73]. The three common mast cell activation pathways are mediated by high affinity IgE receptor FcεRI, G protein-coupled receptors (GPCRs) and Toll-like receptors 2 and 4. The signaling through these receptors triggers degranulation, the best known effector function of mast cells. Although the signaling events that lead to degranulation of mast cells through the FcεRI receptor and TLR2 and 4 are well studied, less is known about the signaling pathways that mediate degranulation through the GPCRs. TLR signaling in mast cells is an exception of the bacterial induced pathways since it does not induce degranulation but instead induce specific cytokine and/or chemokine expression [73]. Moreover, Btk mediated TLR signaling is thought to be closely related in monocyte/macrophage, B and mast cell signaling and the common simplified pathway is showed in Figure 2.

FcεRI

The most extensive studies of Btk's role in mast cells come from Kawakami and colleagues. Stimulation of FcεRI pathways in Btk deficient mast cells causes consequences closely related BCR signaling. For example, Btk deficient mast cells are impaired in PLC-γ activation, IP3 generation and Ca^{2+} mobilization [74]. Btk has been found to co-regulate Kit and FceRI signaling [75]. Moreover phosphorylation of PKB/Akt is dependent on Btk and Syk in mast cells [76]. Downstream effects of Btk are mediated through JNK and p38 and/or transcription factors NF-κB, NF-AT, and AP-1 [76,77], which in Btk defected FcεRI-stimulated mast cells will lead to impaired

IL-2, IL-6, TNF-α and GM-CSF [78,79]. Moreover, after FcεRI cross-linking Btk-deficient mast cells (either *Btk*$^{-/-}$, Xid or Btk knock-down mast cells) showed an impaired histamine secretion and a mild reduction of total leukotriene synthesis *in vitro* [75,79-81].

G protein-coupled receptor (GPCR)

Most of the evidence supporting G-protein coupled receptor mediated Btk activation is based on indirect evidence showing G-protein subunits interaction with Btk [82-84]. Thus, over expression of Gβγ-subunits has been shown to increase Btk kinase activity [84]. Moreover, it has been shown in *in vitro* binding studies that Gβγ can bind to both the PH/TH module and the catalytic domain [85]. Gαq and Gα12 can directly bind and stimulate kinase activity of Btk both *in vitro* and *in vivo* [82,83]. The experiments were done in HEK-293, DT-40 and Meg-01 cell lines, not in primary B or/and mast cells. In paper IV it was found that the novel orphan G protein-coupled receptor 177 (GPR177) was negatively regulated by Btk. This is according to our knowledge the first report which describes specific GPCR regulation by Btk. GPR177 was also up-regulated in Btk deficient transitional type 1 B cells. These experiments were performed with microarray gene profiling and verified with quantitative realtime PCR. The regulation in protein levels needs to be further addressed.

1.1.6 Consequences of Btk mediated signaling

The recently discovered CARD-Bcl10-Malt1 signalosome has been shown to be important for GPCR and mediated activation of NF-κB [86]. It has been suggested that conserved Bcl10-Malt1 complexes interact with different CARD scaffolds to link various receptors, including BCR, TCR, FcεRI, Dectin-1 (denritic cells) and GPRC, to IKK and NF-κB signaling [87]. It will be interesting to see the role of Btk in this context.

Although Btk deficient mice do not generate tumors, the incidence of tumor development is higher in BLNK/Btk double deficient mice, than BLNK single deficient. Moreover, over-expression of a constitutively active form of Btk (E41K) in BLNK/Btk double deficient mice prevent tumor development [88]. Btk's tumor suppressor property is independent of its catalytic activity [89]. In addition, Btk expression is down regulated and in aberrant form in acute lymphoblastic leukemia (ALL) patients [90]. Contradictory to these results, E41K show PH and kinase domain dependent transforming activity in the NIH 3T3 fibroblast cell line [91].

In paper IV, the Btk deficient bone marrow derived mast cells (BMMC) and B cells highly up-regulate melanoma antigen (Mela), which has been found to be related to transforming phenotypes. Mela expression is found in only from wt mice. Human homolog need to be further determined.

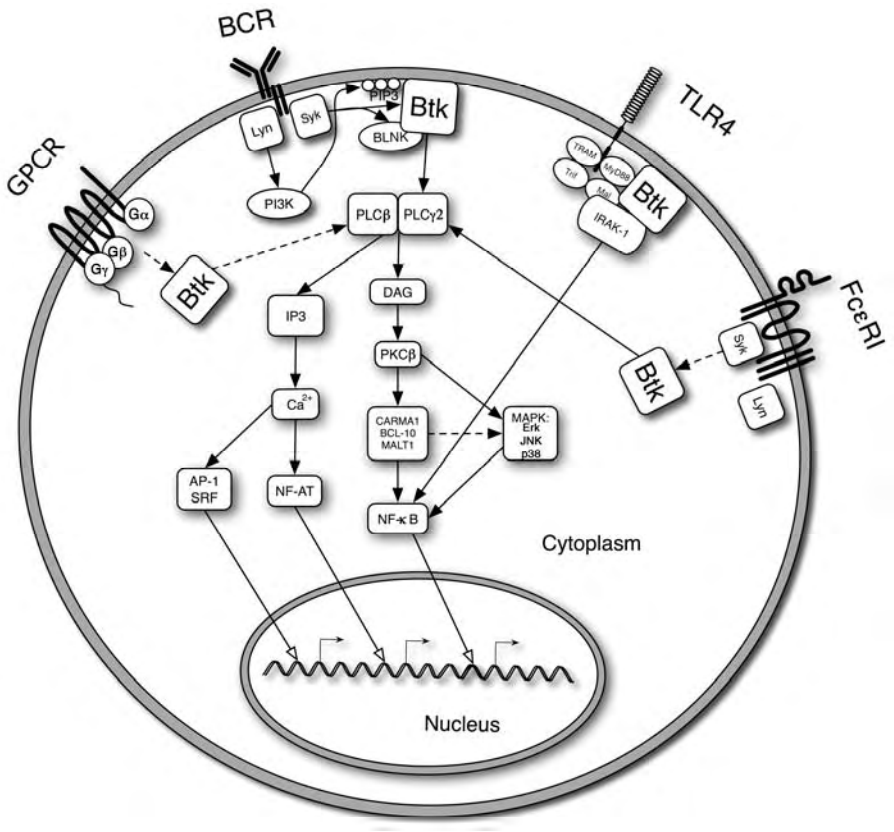


Figure 2. *Common Btk mediated signal transduction in hematopoietic cells.*

1.2 RNA mediated gene silencing

After completion of human genome sequencing, most protein coding genes were thought to be discovered. According to recent understanding there are 20 000-25 000 protein coding genes. These comprise only about 2% of the human genome. The remaining areas have not had any known function and are therefore referred as non-coding regions of genome.

In spite of the discovery that sense and antisense transcription from the same genomic locus seems to be a common event in the human genome, it is unlikely that non-coding RNA could contain unrevealed protein coding sequences since it contains a high density of stop codons and lack any extensive open reading frame [92,93]. Non-coding RNAs can be divided into three classes with respect to their length. RNAs comprising 18 to 25 nucleotides belong to small interfering RNAs (siRNAs) and microRNAs. The Second class is comprised of small RNAs 20–300 nucleotides in length e.g small nucleolar RNAs (snoRNAs), small modulatory RNAs (smRNAs) and Piwi interacting RNAs (piRNAs). These small RNAs are commonly found as transcriptional and translational regulators. RNA consisting of up to and beyond 10,000 nucleotides belong to the medium and large class of RNA e.g Xist and TsiX RNAs, which are involved in mammalian X chromosome inactivation [92]. The medium and large class of non-coding RNAs are involved in DNA imprinting and are produced as precursors for other classes of RNA e.g. microRNA. Owing to the fact that non-coding RNA represents 98% of the human genome, one may speculate that there are plenty more non-coding RNA species waiting to be discovered. Endogenous non-coding RNAs are beyond the scope of this thesis and will therefore not be discussed further. On the other hand the discovery, the function and the utilization of RNA-based post transcriptional silencing (PTGS) tools are to be described concentrating to the use RNA interference (RNAi) in details.

There are four major classes of RNA molecules participating in PTGS: (1) antisense oligonucleotide derivatives that, depending on their type, inhibit translation by steric hindrance or the recruitment of RNase H to cleave the target mRNA; (2) ribozymes and deoxyribozymes - catalytically active oligonucleotides that cause RNA cleavage; (3) small interfering double-stranded RNA molecules that induce RNA degradation through a natural gene-silencing pathway called RNAi; (4) micro RNA that by binding with imperfect complementary to 3’ untranslated regions of mRNA inhibiting translation. The major technological goals for all gene silencing approaches are: resistance to nuclease digestion, good cellular uptake, bioavailability *in vivo*, satisfactory hybridization affinity to the target nucleic acids, binding specificity and low toxicity [94].

1.2.1 Antisense technology

The first description of antisense inhibition of gene expression was revealed when a single-stranded DNA was shown to inhibit the translation of a complementary RNA in a cell-free system in the late 70’s [96]. The discovery was followed by reports about

specific oligonucleotides capable of blocking viral RNA translation of Rous sarcoma virus [97,98]. In general, an antisense oligonucleotide (AON) is a short gene fragment (from 15 to 25 sequence bases) of deoxynucleotides that have a sequence complementary to the target mRNA. The antisense oligonucleotide hybridizes with the mRNA by Watson–Crick base pairing and sterically blocks the translation into a protein [99]. This mechanism is called translational arrest. Another mechanism of AON is the destruction of antisense-mRNA hybrids by RNAase H [100]. The major limitations of antisense technology are the instability of ssDNA oligonucleotides *in vitro* and *in vivo*, the weak intracellular penetration and the poor cytoplasmic delivery, where they have to reach their target mRNA [101].

To improve AON bioavailability, various types of chemically modified AONs have been designed. Most of these chemical modifications are also utilized in RNAi approaches descriebed in next chapter (Fig. 3).

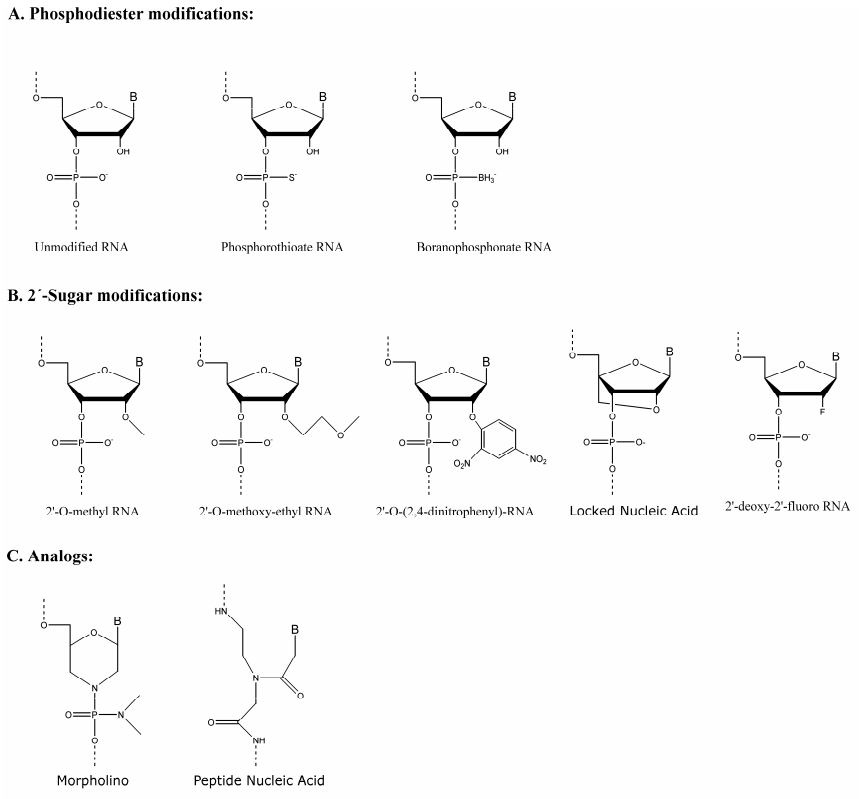


Figure 3. Structure classification of most common nucleic acids and their analogs used in antisense and siRNA gene silencing approaches. Chronologically they can be classified to three groups. The first generation antisense oligonucleotide-modifications, phosphorothioate-oligodeoxynucleotides (PS-ODN), have shown the broadest range of activity in preclinical and clinical studies (ISIS Pharmaceuticals Inc., Genta Inc. and Hybridon Inc.) [101]. The second generation consists of mixed backbone oligonucleotides consists of phosphorothioate modified core with e.g. 2'-Omethyl (OMe) ribonucleosides [104]. The third generation of AONs consists of DNA and RNA analogues such as morpholinos (Avi BioPharma Inc), peptide nucleic acids (PNAs) or at the moment most promising modification lock nucleic acids (LNAs).

1.2.2 Antigene oligonucleotides

Antigene oligonucleotides are antisense sequences that can bind to dsDNA to form a triple helix, and thus inhibit transcription. The first reported antigene molecules were triplex forming oligonucleotides (TFOs) which are able to bind the major groove of DNA and interfere with the transcription initiation and elongation. Typically, the binding caused by TFOs occurs by the polypurine:polypyrimidine tracts by Hoosgsteen pairing [101]. However, these antigene strategies have the following limitations; difficulties to find stabilizing binding conditions for antigene oligonucleotide to dsDNA, need for polypurine or polypyrimidine motifs on target sequences and inefficient nuclear delivery of oligonucleotides [102,103].

Recently, we have shown that by designing Z-shaped, “Zorro LNA”, against dsDNA, it is possible to induce potent sequence specific gene silencing [105]. The antigene effect was more pronounced with Zorro LNA than in sense or antisense strand LNA alone. By using nuclear microinjection we showed effective down regulation of endogenous gene expression. Corey and coworkers have also shown that linear PNA and LNA work as an antigene molecules inhibiting target gene transcription.

1.2.3 Ribozymes

RNA codes for genetic information. However, due to its high flexibility it can adopt high secondary and tertiary structures to obtain enzymatic properties. Catalytic RNAs, Ribozymes, were first discovered by Cech *et al.* in the beginning of 1980’s. The two mainly utilized ribozymes are the hammerhead and the hairpin ribozymes. The hammerhead and hairpin ribozyme RNAs will form thermodynamically favorable tertiary structures. This tertiary structure, which resembles a “hammerhead” or a “hairpin”, allows ribozymes to bind complementary RNA and perform cleavage. The ribozyme mediated cleavage needs the specific sequence in target mRNA which is limiting their usage in gene silencing. Also the complex tertiary structure of ribozymes is limiting the target site entry and has shown to be limiting factor for utilization of this technology. To overcome this, computational or *in vitro* binding assays in the combination of RNase H have been used to predict RNA folding and the target site accessibility [95]. In spite of this, the *in vitro* results poorly represent the *in vivo* situation.

1.2.4 RNA interference (RNAi)

The molecular mechanism of small interfering RNAs (siRNAs) and antisense caused post transcriptional gene silencing (PTGS) is different. Testing identical sequences targeting mRNA in the same mammalian cells, the RNAi mechanism is much more effective than the RNase H mechanism. Moreover, siRNAs were found to silence their target mRNA almost 100 times more effectively than single-stranded antisense PS-ODN [106]. Activation of the endogenous RNAi pathway to down regulate the target mRNA can be achieved in two ways: Either to use vectors to express short hairpin RNAs (shRNA) that resembles miRNA precursors or by introducing siRNAs that mimic Dicer cleavage products into the cytoplasm.

History

Although RNA silencing became the perhaps most important discovery of molecular biology in the late 20th century, the earliest report was published already 1928. In that paper Wingard described how leaves of tobacco plants became resistant to a second exposure of tobacco ringspot virus [107]. Even though, the exact mechanism of this particular virus mediated silencing needed to be further studied, this was the first report that lead to the discovery of RNA mediated antiviral protection or sometimes even called primitive immunity, protection of genome from transposons and regulation of gene expression.

In the year 1990, Napoli *et al.* reported that when over-expressing chalcone synthase responsible for violet coloration in petunias, the outcome was unexpectedly white petunias [108]. This report started the discussion about co-suppression of an endogenous gene with a transgene. RNA silencing was first documented in animals by Guo and Kemphues, who observed that the introduction of sense or antisense RNA to par-1 mRNA resulted in degradation of the par-1 message in *Caenorhabditis elegans* [109]. In 1998 Fire *et al.* published their findings that dsRNA will in fact cause much stronger silencing of target mRNA than ssRNAs separately. Moreover, they described that the phenomenon will be transmitted from the injected animals to their progeny [110]. The concept of RNAi was established.

Even though RNAi was found in 1998, it took three years until it finally became widely used a cross the scientific community. In this finding Elbashir *et al.* reported the first evidence that small interfering double stranded RNAs (siRNAs) can mediate sequence specific gene silencing in mammalian cells [111]. Up until May 2001 RNAi scored 191 hits in PubMed. Today there are more than 9000 hits for RNAi, which is now routinely used in biomedical research.

siRNA

In general, RNAi mediated gene silencing involves three major steps. In the first step, the Dicer specifically hydrolyzes long double stranded RNA or short hair RNA (shRNA) into 21-25nt siRNAs [112,113]. Synthetically produced siRNAs mimic products of this step. In the second step siRNA is incorporated into a protein complex called RNA Induced Silencing Complex (RISC) forming pre-RISC complex which will get activated via ATP hydrolysis and cleavage of sense strand [114,115]. Activated holo-RISC binds to the target mRNA region complementary to the guide (antisense) strand of siRNA and cleaves the phosphodiester bond at position 10 via Argonaute-2 (Ago-2) mediated hydrolysis [112,116,117] (Fig. 5). However, neither the exact mechanism nor, all the proteins involved in these processes are so far from well established.

Dicer

Dicer belongs to the RNase III family and functions as a molecular ruler that measures and cleaves ~25 nucleotides from the end of a dsRNA. The human Dicer contains 7

domains: N-terminal DExD/H helicase, domain of unknown function 283 (DUF283), the RNA binding PAZ domain, RNase III domains a and b, extended inter domain and C-terminal dsRNA binding domain (dsRBD) [118]. A PAZ domain is found in Dicer and in the Argonaute family proteins that are core components of RISC. RNase III cuts in both strands of the dsRNA, leaving a 3' overhang of 2 nucleotide (nt). The siRNA with 2- or 3-nt overhanging 3' ends were more efficient in mRNA knock down than the corresponding blunt ended siRNAs in Drosophila [112,119].

Followed by this original discovery, 2 nt 3' overhang were thought to be necessary for efficient siRNA directed target cleavage. This was followed by the discovery of the ago-2 protein PAZ domain structure, which revealed that the 2 nt 3' overhang is recognized by PAZ domain and is involved siRNA loading into protein complexes active in RNAi [120]. It has also been found that synthetically produced shRNA with 3' overhang serves much more potent Dicer substrates than blunt end constructs [121]. More recently, many different groups have reported that blunt-end siRNA precursor molecules can silence target genes with similar efficiency compared to a conventional siRNA duplex [122]. However, siRNAs with blunt ends are known to activate RIG-1, the sensor for viral dsRNA, and cause off-target effects. It is still not completely clear whether 3' overhangs in siRNA are needed for functional RNAi in mammalian cells.

RISC

RISC has been suggested to have a molecular weight between 140 and 500 kDa [114,123,124]. The elements needed for mRNA cleavage activity in RISC are human ago-2 and the antisense strand of siRNA [116,117]. This suggests that other proteins in RISC may diversify RISC function e.g. substrate turnover or subcellular localization [125]. RISC cleavage produces 5'-phosphate and 3'-hydroxyl termini ends similar to RNase H-type enzyme cleavage products [126]. RISC activity is also dependent on Mg^{2+} ions [127]. The smallest substrate RNA that RISC can cleave is 15 nt [126] whereas a 19 nt duplex has been shown to be the shortest functional size of siRNA. Duplex length itself, but not the base pairing of the antisense siRNA with the target mRNA, seems to determine the minimal length of functional siRNAs. The length of the double-stranded helix is thought to be an important determinant for incorporation into the RISC complex [128]. Once formed, a single RISC complex has been suggested to catalyze the degradation of ~10 target molecules [129].

Martinez and coworkers have shown that substitution of the 2'-hydroxyl group at the cleavage site by 2'-deoxy has no significant effect on target mRNA degradation. Moreover, the catalytic ribonuclease activity or nucleotide base pairing of RISC with the mRNA target site does not involve 2'-OH groups of the guide antisense RNA [130]. On the other hand, substitution by 2'-O-methyl at the cleavage site or all 2' -OH residues of guide strand with -H, substantially reduces cleavage [130], presumably due to steric hindrance [126]. To support this model, an A-form helix formation has been found to be necessary between target mRNA and guide strand [131]. In conclusion, the limiting factor for successful siRNA function is possibly related to RISC target

accessibility. The degradation of mRNA by RNAi occurs in the cytoplasm. The more specific location has been suggested to be the Cytoplasmic processing bodies (P-bodies). However, according to the recent work of Eulalio *et al.*, P-bodies are not required for siRNA or microRNA mediated silencing pathways. The authors showed that by blocking the siRNA or miRNA pathways P-body formation is prevented, indicating that P-bodies arise as a consequence of silencing [132].

Design Rules

From the first day of discovery of RNAi in mammalian cells, the most frequently asked question related to RNAi has been how to design a functional siRNA duplex. A number

of academic and commercially affiliated web-based algorithms and programs have been developed. These tools are based on solely computational algorithms, experimental data or their combination (table 1.). However, the complete rules that fully describe the efficient siRNA-directed silencing are still unknown. Up-to-date rules for designing of siRNA/shRNA are presented in the table 2.

Table 1. *Internet-based siRNA design sites.*

Web-based non-commercial programs for siRNA design:
RNAi Central, Hannon Lab:
http://katahdin.cshl.org:9331/RNAi_web/scripts/main2.pl
BIOPREDsi, Novartis Institutes for BioMedical Research:
http://www.biopredsi.org/start.html
siR, University of Texas Southwestern Medical Center:
http://biotools.swmed.edu/siRNA/index.htm
siDirect, University of Tokyo:
http://design.rnai.jp/
siSearch, CGB at Karolinska Institutet:
http://sirna.cgb.ki.se/
The human siRNA database (HuSiDa):
http://www.human-siRNA-database.net
RNAi Codex, a single database that curates publicly available RNAi resources.
http://rnaicodex.cshl.edu/scripts/newmain.pl
Extensive collection of commercial and non-commercial algorithms and sequence prediction programs for RNAi:
http://inn.org.il/workshops/huji/siRNA-links.html

Table 2. *Design Rules (to obtain the most optimal siRNA/shRNA).*

siRNA/shRNA Sequence
1. *A*, more preferably, or *U* at the 5'-end of the antisense strand.
2. *A* at position 3 and *U* at 10 of the sense strand.
3. *GC*-content of the siRNA strand has been shown to be optimal between 36-52 %.
4. Start evaluation of siRNAs efficiency with 3-5 different sequences.
Size
Can vary between 21-30 nt. However, 25-30 nt sequences have been shown to be more potent as compared to conventional 21 nt.
shRNA stem length
Can vary between 19-29 nt. It is also possible to include an miRNA-like 27 nt leader sequence.
shRNA Loop
Loop size can vary between 4 to 23 nt. 9nt loop has proven optimal in many applications.
Factors to avoid
1. Regions within 50-100 bp from the start and the termination codon. Moreover, sites for mRNA-binding proteins in the exon-intron boundaries.
2. G/C at the 5'-end of antisense.
3. G at position 13 of sense.
4. Intron regions.
5. Stretches of 4 or more bases such as TTTT and GGGG.
6. Repeats and low complex sequence.
7. Single nucleotide polymorphism (SNP) sites.
8. Sequences with partial homology of non-target mRNA.
9. GU-rich sequences e.g. GUCCUUCAA and UGUGU. GU-rich sequences are often immunostimulatory.
10. Long ≥ 30nt dsRNA can activate PKR response.

siRNA function and optimization

The opening of siRNA duplex from 5' antisense end by G:U mismatches has been shown to increase the efficiency of siRNA. Moreover, the thermodynamically higher base paring at 5' end of siRNA duplex has been suggested to favor antisense strand loading into RISC and therefore increased siRNA functionality [133,134]. We and others have tested this hypothesis by designing mismatches into siRNA duplexes. The siRNA duplex was opened with G:U mismatch inserted into 5'-prime of antisense strand. However, this did not increase the efficiency of already highly efficient siRNA construct [25]. Neither did the opening of the 5' sense strand decrease the down regulation efficiency. These results indicate that finding an optimal target sequence may play a more important role than one nucleotide end opening in the siRNA designing.

siRNA has been shown to down regulate target mRNA as early as 6 h post transfection. Maximal down regulation is achieved between 42-54 h post transfection [131]. The siRNA amplification process found in flies and lower eukaryotes, is not thought to occur in mammalian cells because of at least two reasons: Firstly, long dsRNA is the

substrate of Dicer and will be cleaved to 21-23 nt siRNAs. However, long dsRNA activates interferon responses in mammalian cells, and thus, barely exists in a similar manner as in lower eukaryotes. Secondly, the RNA dependent polymerase (RdRP), found in lower eukaryotes but not in mammalians, uses siRNA as a primer, creating dsRNA from mRNA [135]. Since RdRP also requires a free 3'-OH group, which is not needed for RNAi, it is likely that RNAi in mammalian cells does not occur through a RdRP dependent degenerative PCR mechanism [131,136,137]. The effective siRNA duplexes produce potent silencing at 1-10 nM concentration [137]. The higher the dose, the greater the risk for unspecific effects. The optimal time for siRNA to reach its highest down regulation capacity depends on the nature of the experiment. Targeting of the endogenous expressed gene requires longer time than co-transfection experiments, which is due to the target protein stability. It has also been reported that low-abundant transcripts are less susceptible to siRNA-mediated degradation than transcripts of medium- and high-abundant [138].

Single stranded RNA molecules will be rapidly hydrolyzed by RNases inside the cells. siRNAs designed as double stranded short RNA molecules are more resistant to nucleases. Once loaded into the effector-RISC complex, the antisense strand is, despite the possible protection by the RISC complex proteins, vulnerable to degradation. By introducing chemical modifications to siRNA, similar as in antisense oligonucleotides, it is possible to stabilize and prolong the siRNAs kinetics *in vivo*. When introducing chemical modifications into siRNA it is important to understand the vulnerable sites for siRNA function. Mismatch tolerance has been shown to be position and nucleotide dependent. Generally, there is a lower tolerance for mutations located in the center of siRNA [139,140]. Conjugations in the 5' and 3'-prime end of the sense and the 3'-prime of the antisense strand have no effect, where as conjugations in the 5'-prime of antisense abolishes siRNA function [128,131]. A phosphorylated 5'-antisense strand of siRNA is required for RNAi *in vivo* in human and Drosophila cells [114,131]. These and other studies have also shown that the sense siRNA strand is frequently more tolerant to chemical modifications than the antisense siRNA strand, indicating different requirements for assembly of RISC and targeting by RISC [126].

LNA has been shown to confer both high affinity as well as nuclease resistance when present in antisense oligonucleotides. LNA substitutions in the siRNA duplex have been shown to reduce off-target effects and increase stability [141,142]. However, the modifications also reduced the capacity of target knock down. Modifications summarized in figure 3 are also utilized for stabilization of siRNAs. These will be further discussed in chapter off-targeting. .

It has been reported that 27-29 nt siRNA is up to 100 times more potent of inducing target mRNA degradation than conventional 21nt siRNAs [121]. These long siRNA have been called Dicer substrates, based on the fact that Dicer will cleave them first to shorter siRNA, which are then loaded to RISC. Since Dicer is involved also in loading siRNAs to RISC it has been proposed that to attract Dicer more efficiently and thereby cause more potent gene silencing one should design longer siRNA or shRNA

[121,143]. Endoribonuclease-prepared siRNAs (esiRNAs) are a novel approach to produce large amounts of siRNA for screening experiments [144]. However, the application of synthetic siRNAs is restricted to short-term persistence of transient expression. Moreover, a single transfection of siRNA may not provide a sufficient window of functional depletion for proteins with long half-lives [145]. This can be overcome by using endogenously shRNA producing plasmid vectors.

Delivery of siRNA

The general hurdle of gene therapy has been the delivery of therapeutic agents into cells and further to their target. Initially, the delivery of siRNA into cell lines was carried out with conventional methods used also for oligonucleotide and plasmid delivery. These methods include chemical methods, e.g calcium phosphate, commercial cationic lipid or non-lipid based reagents and physical methods e.g. microinjection and electroporation.

Oligofectamine™ from Invitrogen was one of the first successfully used lipid composed reagents used for siRNA delivery *in vitro*. Oligofectamine has the advantage of being non-toxic to cells so change of medium after transfection is therefore not needed. One interesting method for high-through-put assays is reverse transfection method [146,147]. A reverse transfection involves the deposition of lipid–nucleic-acid complexes on a solid surface, often a glass microarray slide. Cells tat are plated on top of the slide, take up the encapsulated RNA causing target gene silencing. Another conventional method used in siRNA delivery is electroporation and one of its commercial application Nucleofection™ by Amaxa Biosystems. These methods are more suitable for primary cell transfections than lipid reagents [148].

Systemic delivery

A short while after the invention of RNAi suitable for mammalian cells, the speculations of its therapeutic potential began. The common limitations of all RNA mediated gene expression silencing and their therapeutical applications have been the delivery into the body and cellular the uptake. The first *in vivo* trial was done in mice and described the potential of hydrodynamic injections of naked siRNA against Fas [149]. Biodistribution of siRNA's is the same as for single-stranded antisense agents and requires large and repeated dosages for efficacy [150,151]. Naked siRNA has been reported to require chemical stabilization for *in vivo* use [152,153].

However, naked and unmodified siRNA is prone to rapid degradation by nucleases. The 5'-end of the sense strand and both 3'-ends can be used for attachment of functional groups bearing fluorescence [43, 44] or radioactivity [73] as well as groups facilitating cellular uptake and transport through membranes (e.g. cholesterol, litocholic acid, lauryl acid or long alkyl chains) [74, 93, 94]. In general, modifications located at the 3'-ends of the sense and antisense strands as well as at the 5'-end of the sense strand are accepted to maintain siRNA silencing activity. A phosphate group at the 5' terminus of the antisense strand is required for a functional siRNA duplex.

The discovery of siRNAs potential as a therapeutic drug was followed by vast numbers of applications, many of them based on lipid encapsulation of siRNA and backbone modification to increase the stability and cellular uptake. Stabilized Plasmid Lipid Particles (SPLP), consisting of lipid bi-layer encapsulating a single copy of plasmid DNA, have been reported as a potential method of systemic nucleic acid delivery [154,155]. The method has also been utilized for siRNA delivery. Chemically stabilized siRNA was incorporated into a specialized liposome complex referred as a stable-nucleic-acid-lipid particle (SNALP) [155,156]. The SNALP bi-layer contains a mixture of cationic and fusogenic lipids that enable the cellular uptake and endosomal release of the particle's contents. Morrisey *et al.* demonstrated the potency of SNALP siRNA to decrease the hepatitis B virus levels in mouse serum. Moreover, SNALPs were used to target apolipoprotein B in non-human primates [157]. Polycation polyethylenimine (PEI)-complexed siRNA has been a vastly used approach in systemic delivery of siRNA [158-160].

Targeting

To increase target specificity, aptamers have been used in combination with siRNAs. Chu *et al.* demonstrated that by forming an aptamer:streptavidin:siRNA conjugate it was possible to deliver siRNA to prostate cancer cells *in vivo* [161]. These authors also demonstrated that the 5' sense strand of siRNA can be conjugated with targeting ligand/moiety without affecting its efficacy. In addition, self-assembling nanoparticles have been used as an alternative approach to deliver siRNA. PEGylated polyethyleneimine (PEI) combined with integrin-binding arginine–glycine–aspartic acid (RGD) peptide has been used successfully to direct siRNA uptake to cancer cells [159]. Another related application described is the usage of cyclodextrin containing polycation siRNA complex conjugated with transferrin to target transferrin receptor expressing tumour cells [162]. In addition, non-covalent complexation of siRNA with protamine-Fab antibody fusion protein targets siRNA to cell specific receptor-mediated uptake. This has been shown to inhibit HIV replication in primary T cells as well as targeting siRNA to ErbB2-expressing cancer cells [163]. Recently, 9 residues of D-arginine, cell penetrating peptide, were added to the C-terminus of the rabies virus glycoprotein (RGV). This positively charged peptide and negatively charged siRNA formed a complex and entered across the blood-brain barrier targeting neuronal cells specifically [164]. The drawback of this method was poor silencing capacity reaching at most to 50% down regulation *in vivo*. Authors speculate that this might be due to the degradation of unmodified and unprotected siRNA. Another reason might also be the poor loading of siRNA into RISC from positively charged peptide complex.

1.2.5 shRNA producing vectors

Soon after the discovery of siRNA, it became clear that a transient siRNA effect was not sufficient for all applications e.g. targeting proteins with a long half-life. Another potential weakness of chemically synthesized siRNAs is the variability in transfection, especially in difficult-to-transfect cells. To overcome these problems, constitutively

expressing short hairpins RNA (shRNA) vectors were developed. shRNA, siRNA and micro RNAs (miRNA) can be produced from plasmids containing Pol II or Pol III class promoters. Products of these vectors resemble pre-miRNAs and undergo Dicer processing. shRNA vectors contain 19 to 29 nt homologous sequence complementary to target mRNA. The shRNAs produced by these vectors are further processed to functional siRNAs and uses RNAi to degrade the target mRNA like synthetic siRNAs (Fig. 4).

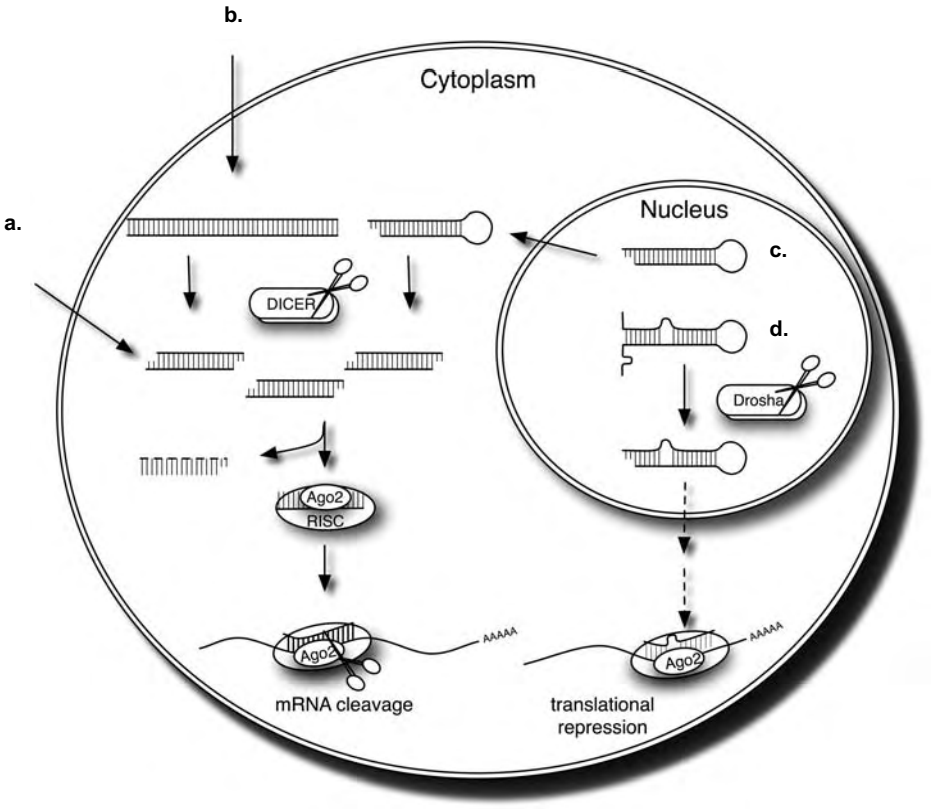


Figure 4. *RNAi pathways; a) siRNA, b) dsRNA, c) shRNA and d) miRNA. Briefly; dsRNA, siRNA and shRNA share common pathway after Dicer recognition. These RNAs are processed to siRNAs which will be loaded to RISC. The sense strand will be removed and target mRNA will be cleaved. miRNA, on the other hand, will be first processed in the nucleus by Drosha, Dicer like nuclease, followed by export to cytoplasm. In the cytoplasm it will also bound to RISC, but because of partial homologues sequence, and mediate translational repression.*

Among these, the first and most frequently explored vectors are based on Pol III promoters. Less than one year after discovery of siRNA in mammalian cells two independent groups described Pol III promoter-driven shRNA plasmids. Brummelkamp *et al.* cloned the H1 promoter containing pSUPER vector, which was the first shRNA producing vector [165]. pSUPER and its follow-up retroviroid pSUPER_Retro became one of the most referred vectors in shRNA utilizing research. Paddison *et al.* developed the first long 500 nt RNA expressing vector containing a U6 promoter [166]. Shortly

after its discovery the vector was modified to produce 21-29 nt long shRNA and was named pShag [167]. In addition to these two promoters, other Pol III promoters such as 5S, 7SK and tRNA promoters were developed to stably express shRNA in mammalian cells [168-170].

Classification of shRNA vectors

Generally shRNA can be classified in to three main groups according to the strategy for creating shRNA inserts. Inducible shRNA vectors, discussed in the next sections, are excluded from this classification. The most commonly used method requires shRNA-insert synthesis, annealing and ligation into vector [171,172]. The second strategy uses PCR in which primers are bound to both sides of the promoter [173]. The 3'-primer contains a ss hairpin sequence. This system has problems stemming from secondary structures of the 3' primer. The advantage compared to the first strategy is the lack of truncated small shRNA oligos that are causing background problem and need to be removed with PAGE purification. The third and least used method encompasses several techniques relating to primer extension where polymerase extends the 3' end of overlapping oligos [174,175].

Another classification of shRNA vectors can be done according to their production of siRNA *in vivo* (Fig. 5). There are vectors which contain promoters for both sense and antisense strands. Following transcription, these separately expressed ss RNA strands will hybridize to form siRNA duplex *in vivo*. The second, and most common type of shRNA vector, is a variant where Pol III promoter is followed by the shRNA oligo and five thymidine serving as stop codon for Pol III promoters. The third types of vector contain a Pol II promoter and minimal polyadenylation signal. These vector have been tailor made to contain a selection marker gene e.g. EGFP that will be transcribed from same promoter. These vectors can also express imperfect pairing duplex structures that are based on pre-microRNA structures. The development and usage of these types of vector libraries have become more popular after the discovery of endogenously expressed microRNAs in the mammalian genome. Pol II

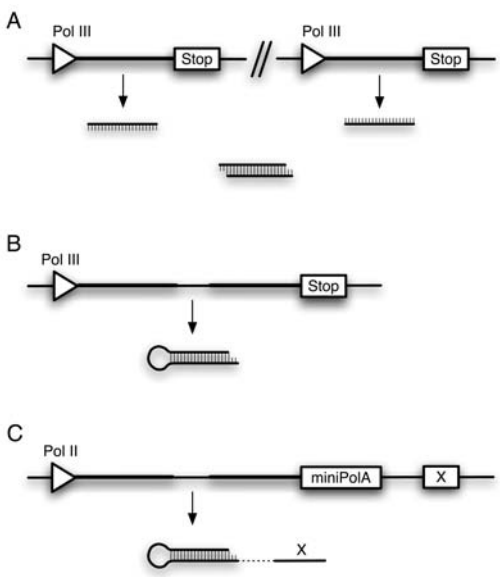


Figure 5. *Functional classification of shRNA vectors. A vector contains separate Pol III promoters for sense and antisense strands of shRNA which will anneal together in vivo. B vector is the typical shRNA vector containing Pol III promoter and five thymidines as a stop codon. C vector is a shRNA vector with Pol II or Pol II fusion promoter and minimal polyadenylation signal. The x can be e.g. selection marker gene.*

promoters have also been used for vast shRNA applications. However, this is subject to certain constraints. For instance, some authors have demonstrated a need for both a very short distance (6 bp) between the Pol II promoter and the shRNA sequence as well as a minimal polyadenylation signal for functional shRNA production [176,177]. This is not the case for Pol III, where we have shown that there can be over 40 nt gap between the promoter and transcription initiation site [177,178]. Others have shown that the presence of an intron between the Pol II promoter and the shRNA sequence is necessary for efficient production [179]. On the other hand, the Ubiquitin C promoter has been shown to drive efficient shRNA expression when the shRNA cassette has been inserted inside the intron to mimic miRNA (miR-30a) [176,180]. In Pol II promoter constructs the shRNA cassette can be flanked by a reporter gene, e.g. EGFP, which allows quantification of shRNA expression. Another advantage of Pol II promoters is the possibility to create tissue specific expression of miRNA or shRNA. The advantages of Pol III promoters for shRNA production are the high level of activity and the tight regulation. It has been estimated that there are on average more than four times more Pol III produced transcripts compared to Pol II in Hela cells [181]. Moreover, Pol III does not add extra sequences that might affect the functional activity of shRNAs [182,183].

Multiple modifications from these three main types of vectors have been developed. Paul and colleagues showed that using the U6 promoter and the first 27 nucleotides from the U6 snRNA there was more potent down regulation capacity compared to the U6 promoter followed directly by a hairpin [184,185]. Xia *et al.* have shown that by introducing an enhancer from the cytomegalovirus immediate-early promoter in close proximity to the U6 promoter will increase the expression of shRNA [186]. Lately it has been shown that a hybrid CMV enhancer minimal promoter/H1 promoter construct might be a promising candidate for specific targeting *in vivo*, e.g. expression in the mouse brain [187]. Today, there are tens, if not hundred's of different versions of shRNA vectors utilizing retroviroid, lentiviroid or adenoviroid backbones. shRNA vector libraries targeting the majority of human and mouse genes have been developed [188]. The most recent development of shRNA vectors are microRNA vector libraries [189].

1.2.6 Inducible shRNA vectors

Utilization of shRNA producing vectors against genes that are vital for cellular homeostasis is not feasible. In the optimal case these types of experiments would require viral shRNA vector for efficient cellular uptake, and tightly temporally controlled as well as cell specific inducible shRNA production. In order to achieve these expectations, multiple different inducible shRNA vectors were created. The concept of inducible promoters is not unique for shRNA plasmids. Most of the systems have been obtained from the Pol II controlled vectors developed for inducible gene, antisense or ribozyme expression. Inducible systems are mainly build-up from tetracycline-, ecdysone- or Cre-recombinase/LoxP-based vectors.

Tetracycline based systems

It is well established that pol II transcription can be exquisitely regulated in cell culture or *in vivo* by using tetracycline (tet) based doxycycline inducible/repressible promoters [175]. Most of the tet-inducible systems are built from two separate vectors: The first vector containing U6 [168,190,191] or H1 [178,192,193] Pol III promoter in close proximity to the tetracycline operator followed by the shRNA cassette. The second vector contains chimeric transcription regulator containing tetracycline repressor (TetR) fused to transcriptional activator domain. They are named Tet-on or Tet-off according to their nature of induction of shRNA production. In the cell expressing both plasmids, chimeric TetR is bound to operator inhibiting the shRNA transcription. Doxicyclin added to cells will bind to free TetR protein and compete out the TetR binding to operator initiating shRNA production. The bottle-neck for tetracycline-based methods has been the basal promoter activity without doxycyclin. Moreover, the usage of multiple separate plasmids has been considered to be a drawback shared between all inducible vector systems.

Recently developed a two-plasmid tet-on system contains plasmids for T7 polymerase and a self-cleaving ribozyme-shRNA fusion construct expression. This system uses the highly specific T7 phage RNA polymerase, not endogenously expressed in mammalian cells, limiting the possible basal transcription of Tet-on systems. The first plasmid is responsible for doxycycline-dependent expression of T7 RNA polymerase while the second plasmid expresses shRNA-ribozyme fusion transcript under the control of a T7 promoter [194]. After doxycycline induced T7 polymerase production the shRNA-ribozyme fusion transcript is produced. This is followed by ribozyme mediated cleavage of fusion transcript releasing functional shRNA. The advantage of this system is the possibility to engineer 3' ends of shRNA, which in the case Pol III systems is solely uridine residues.

Szulc *et al.* reported the generation of an inducible tetracycline system based on a single plasmid utilizing the DNA-binding protein KRAB fused to TetR and H1 Pol III promoter [193]. However, the basal read-through from the repressed promoter is between 4-9 %, depending the amount of plasmids transduced in the cells [193]. This fundamental problem of tetracycline-based methods remains unimproved. The tet-on system also has a problem in doxycyclin responsiveness during the development of transgenic animals, and therefore is not beneficial for *in vivo* approaches (Herold, MJ personal communication).

Ecdysone based systems

The ecdysone-based inducible vector system is constructed from three different vectors containing: 1. retinoid X receptor (RXR), 2. Modified ecdysone receptor (VgEcR) and 3. ecdysone-reponsive elements (5xE/GRE) flanked with DNA-binding domains, minimal heat-shock promoter (ΔHSP) followed by the shRNA cassette. Followed by an introduction of ecdysone analogue e.g. myristerone A, the 1 and 2 receptor heteromerize and bind to the DNA-binding elements of the third vector thus activating

shRNA production. By using this system Gupta *et al.* showed reversibly inducible knock down of p53 [195].

As all the other inducible systems, the ecdysone-inducible has some limitations. To start with, the function of the ecdysone inducible system has been shown to be restricted for limited number of cell lines. In addition, the system contains three plasmids and one chemical drug, making it a complex and time consuming approach especially in *in vivo* use. However, this and other systems are commercially available with an optimized protocol, making those more user friendly. The advantage of using the ecdysone system is the lower background expression compared to tetracycline-based systems [196].

Cre-LoxP based systems

The third inducible system is based on site-specific recombination orchestrated by Cre-recombinase. Cre-recombinase, as well as its target LoxP sites, are extracted from the bacteriophage P1 genome. They provide a cross-reaction free system when introduced in the mammalian cells. The Cre-LoxP system is widely used in generating transgenic animals and can therefore be considered free of any major toxic effects. Generally, Cre-LoxP-systems are built on two vectors. The first vector contains Pol III-promoter, followed by LoxP-Stop(five thymidines)-LoxP-stuffer cassette and shRNA sequence respectively. The second plasmid is for the Cre-recombinase production.

The 34-bp LoxP site consists of two 13 bp inverted repeats separated by an 8 bp spacer [197]. These repeats are the Cre binding sequences, and depending on their orientation, they are steering the recombination process. LoxP sites are highly specific sequences, allowing only 2 bp modifications of the most distal part of the inverted repeats without altering recombination frequency [198]. The differences between the Cre-LoxP systems are the placement of LoxP in respect to the Pol III promoter, the shRNA cassette and the existence of reporter gene. Moreover, Cre-LoxP approaches differ from each other by their method to introduce Cre into the nuclei.

The LoxP site has been designed into the U6 promoter, partially replacing the LoxP spacer with a TATA-box [199,200]. However, a modified LoxP site exhibits only ~35 % activity compared to wild type levels [199]. Fritsch *et al.* and, shortly thereafter Kasim *et al.* cloned the neomysin resistance gene with a Pol III stop codon flanked by LoxP sites in the shRNA loop sequence [201,202]. Inactive vector produced only sense strand of shRNA and neomycin promoter-gene. These cells could be selected with neomycin until the shRNA expression was initiated. The Cre mediated recombination resulting shRNA containing a 34 nt LoxP loop. However, this long shRNA loop, caused inefficient down regulation, depleting at most 50 % of the target gene expression [201]. In Paper III a stuffer cassette was introduced between H1 promoter and the initiation codon (Fig. 9). The advantage of this approach was the efficient length of shRNA without any additional sequences expressed. The stuffer cassette consists of five thymidine residues serving as a Pol III stop codon, and a CMV-promoter-EGFP reporter gene flanked by LoxP sites [178]. A Lentiviral inducible

(pLIND) expresses GFP in its inactive state. Followed by the introduction of Cre, EGFP expression is lost and shRNA production is initiated. In order to track active shRNA producing plamid *in vivo* we have added elongation factor alpha promoter in frame with enhanced yellow fluorescent protein- reporter cassette (Fig. 6)

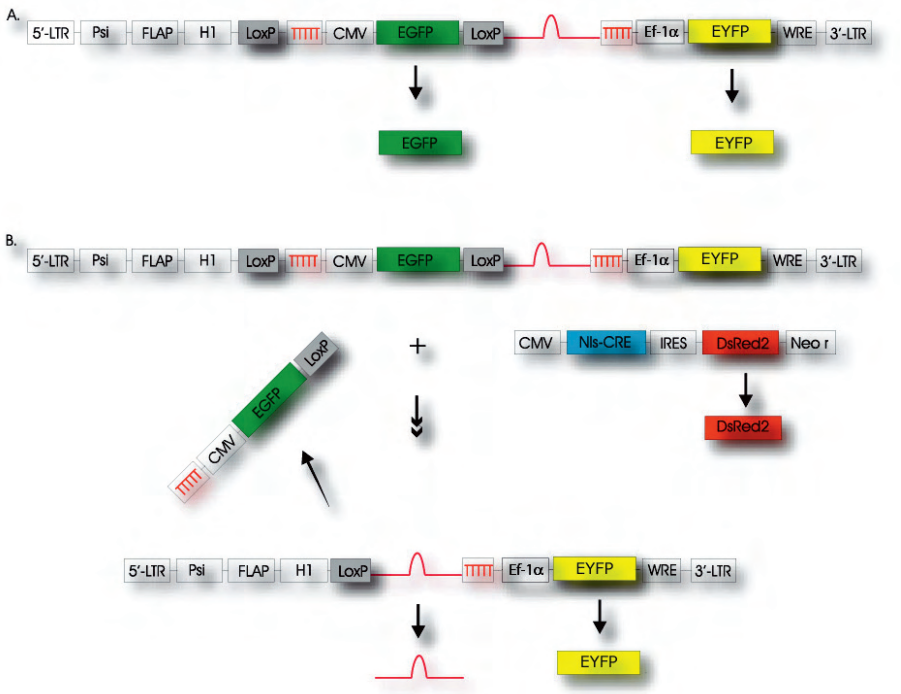


Figure 6. *Schematic representation of Cre-LoxP vector system pLIND$^{in\ vivo}$ (Heinonen et al. unpublished results). (A) The pLIND$^{in\ vivo}$ plasmid alone allows the EGFP stuffer reporter cassette and EYFP to be expressed under the CMV and Ef-1a promoter respectively. The difference between pLIND and pLIND$^{in\ vivo}$ is the Ef-1a-EYFP cassette inserted into latter. The shRNA remains unexpressed, as the H1 promoter is not functional due to the presence of a termination sequence (five thymidines) after the first LoxP site and two clusters of four repetitive thymidines inside the CMV promoter. (B) After the expression of pNLSCRE/IRES2/DsRed2 plasmid, the CRE will cause LoxP recombination, which will remove the EGFP stuffer cassette, and the shRNA will be expressed. The insertion of Ef-1a-EYFP cassette into pLIND$^{in\ vivo}$ allows the detection of shRNA containing cells.*

Cre recombinase

The common challenge for Cre recombinase-based systems is the delivery and introduction of Cre into cells; in other words, how to induce the inducible shRNA production. Many attempts have been made in order to find an optimal method for Cre production, including fusion proteins with nuclear localization signal (nls) and/or TAT from HIV produced and purified *in vitro* [202,203], cell type specific promoter containing vectors [204] and tamoxifen-inducible Cre-recombinase fused to a mutated ligand-binding domain of the human estrogen receptor [205]. We have recently developed a Cre induction system based on exogenous control of mammalian gene expression via modulation of translational termination originally introduced by Murphy

et al. [206]. Following introduction of a stop codon into the Cre coding strand one can efficiently inhibit translation. This can be reverted by introduction of aminoglycoside antibiotics, which induce Pol II promoter read through (Heinonen *et al.* manuscript in preparation). Inducible systems utilizing exogenous controllable Pol II-promoters have been developed. Unwalla *et al.* have created an HIV-1 TAT inducible fusion promoter containing HIV-1 LTR and minimal heat shock promoter hsp70 from *Drosophila melanogaster*. The authors demonstrate shRNA expression from Pol II fusion promoter only when cells were infected with HIV-1 [174]. However, the system still exhibits poor shRNA expression, a common weakness of Pol II promoter mediated shRNAs expression.

Inducible shRNA vectors represent the most advanced tool of siRNA application for cell signaling research. The reversibility is the unique advantageous feature of the Tetracyclin-based shRNA expression systems. The fact that there is no perfect inducible shRNA system underlines the importance of specifically choosing the most optimal system for each specific application. In the future, the optimal shRNA expressing vector would contain reversibly inducible chimeric Pol III/Pol II promoters expressing shRNA quantitatively and tissue specifically. Since the use of viral vectors in clinical applications carries a risk of random integration into chromosomal DNA and thus poses a serious threat to the therapeutic safety [207], other delivery possibilities need to be considered.

1.2.7 Challenges in shRNA vector production

Compared to siRNA, shRNA and inducible shRNA vectors offer advantages in silencing duration, effective viral delivery and cost. However, the construction of shRNA vectors poses technical challenge caused by the palindromic hairpin structure. It has been suggested that shRNA constructs get mutated within the hairpin region [145]. One possible explanation is that commonly used *E. coli* strains such as *DH5α* encode sbcC and sbcD nucleases, which are known to generate double-stranded breaks in DNA hairpin structures [208]. This problem can be circumvented by using a sbcCD engineered *E. coli* strain such as *GT116* (Invivogen) or recombination-deficient *SURE2* (Stratagen).

1.2.8 Micro RNA (miRNA)

The discovery that the *lin-4* gene, known to control timing of larvael development in *C. elegans,* does not code for protein but instead produces a pair of small RNAs, can be considered the first characterization of an RNAi-like phenomenon in animals [209]. *Lin-4* was shown to bind 3' UTR of *lin-14* gene, reducing the lin-14 protein amount. The best known endogenous regulatory RNAs involved in RNAi in mammalians are microRNAs (miRNAs). At the moment there are estimated to be over 600 miRNAs in the mammalian genome. The generation of miRNAs occurs via sequential processing and maturation of long primary transcript (pri-miRNA). Drosha RNase will cleave Pri-miRNA into pre-miRNA in the nucleus. After transportation to the cytoplasm pre-miRNA will be cleaved by Dicer into mature 22 nt miRNA. Mature miRNA will be loaded into RISC. RISC containing the miRNA guide strand will then bind via

miRNAs imperfectly complementary to 3'-UTR of mRNA and cause translational inhibition (Fig. 5) [210]. The deeper understanding and exploitation of the miRNA is in the interest of many future biomedical applications.

1.2.9 Off-targeting

RNAi is a powerful method to down regulate gene expression. However, its utility will depend on its target specificity. One of the outcomes of indifferent design is mistargeting of siRNA, often called off-targeting. Both siRNA and shRNA have been reported to cause off-target down regulation [211]. Off-targeting can be divided to two main disciplines. The first causes down regulation of unintended genes which share sequence homology with siRNA. The second off-target type is the PKR-interferon response which is triggered following dsRNA exposure. In general, siRNAs are thought to be short enough to by pass the dsRNA-induced interferon effects in vertebrates. The IFN response represents an early host defense, one that occurs prior to the onset of the adaptive immune response. The best-characterized contributors to the interferon-induced response are PKR and oligoadenylate synthase (OAS). Following introduction of dsRNA into the cell, PKR and OAS can get activated, causing translational inhibition and RNase L mediated mRNA degradation respectively [212]. In fact, the 21 nt siRNA can activate PKR in a concentration-dependent manner *in vitro* [213]. Since naked siRNAs injected into mice were not causing an interferon response, this response might not only be dependent on siRNA sequence but also on the delivery complex, where its bound [214].

The first sign of a possibility that off-target effect might exist in siRNA was published by Boutla *et al.* where they reported that mutations in the middle of siRNA only moderately abrogate the down regulation capacity [215]. It has been shown that 2'-OH groups of uridines are associated with immune recognition of siRNAs. Single-stranded siRNAs are more immunostimulatory than their respective double-strand siRNAs. Also, blunt structure at the 3' end is the strongest terminal structure for promoting activation of dsRNA-dependent PKR [145].

Recently, Jackson *et al.* among others showed that many off-target genes had sequence similarity to the 3' UTR regions of the transcripts [216-218]. Moreover, they found an 8 nt conserved region from siRNAs 5' antisense strand, being the most important region to off-targeting. Because off-target silencing apparently requires only 7 nt complementary it can be assumed that most siRNAs and shRNAs have an individual cross-silencing signature [216,218-220]. Therefore, it is extremely difficult, if not impossible, to design siRNAs that will not cause off-target effects. This off-target regulation by siRNAs and shRNAs is reminiscent of target regulation by miRNAs.

How to avoid off-targeting

It has been shown that both sense and antisense strands of siRNA can induce gene silencing [134,138]. By introducing mismatches to the sense strand, it is possible to decrease the sense strand originated off-target effects [133]. The regulation of the

imperfectly matched targets is a fundamental challenge of siRNA and shRNA function. Pooling of siRNAs has been suggested to be the most promising way to minimize the off-target effects [217,219-221]. This can be explained by the lower concentration of individual siRNAs needed in a pool that spreads out the effect of individual off-target seed sequences, if optimal, below the threshold of gene silencing. Each single siRNA duplex in a pool has different off-targets while having the same on-target. The use of siRNA pools dilutes off-target effects [219]. siRNA off-target transcripts also tend to contain longer 3' UTRs than background transcripts. Importantly, the siRNA off-target transcripts have been shown to be enriched in miRNA target sites [220].

An ssRNA sequence containing poly(U) or (GU)-rich sequences has been shown to have immunostimulatory properties that are mediated through toll-like receptor 7 (in murine) and 8 (in humans) suggested that siRNA duplexes might be ligands of toll-like receptor 7 or 8 [222,223]. The GU-rich sequence 5'-UGUGU-3' has been reported to be specific for siRNA induced immunostimulation [224]. By introducing 2'OMe modifications to 5% of guanosines or uridines within one strand of the duplex the immunostimulatory activity of siRNA, irrespective of its sequence, is fully abrogated [225].

Off-target effects can also be substantially reduced by chemical modifications. siRNAs can be modified to cause less off-target effects and more nuclease resistance by replacing the 2' OH group with 2'O-methylation, 2'H or 2'F modification [226]. By introducing the 2'O-Me modification in the position 2 of the guide strand reduced off-target effects dramatically without compromising on-target silencing potential [227]. However, the chemical modifications of siRNA will create new synthetic oligonucleotides not found in nature. This will raise an additional safety concern for clinical trials. Another fact to consider is how long siRNA is needed to be active in the blood stream. Efficient delivery inside the cells might be enough for successful siRNA therapy. In addition, off-target effects can be by minimized using a computational software tool designed by Xu *et al.* and proper experimental set-up [228].

1.2.10 siRNA in Therapeutics

If siRNA has been widely utilized in basic research, it has also become popular for drug development. Alnylam Pharmaceuticals, Inc. is developing siRNA based therapies against viral infection and metabolic disease. The siRNAs have been administrated by direct inhalation to treat Respiratory Syncytial Virus (RSV) infection. Moreover, they are in the process of developing a small interfering RNA molecule that reduces the production of VEGF named, Bevasiranib (Cand5). This compound is utilized for the treatment of ocular diseases including diabetic macular edema and exudative age-related degeneration. The Bevasiranib clinical trials are in phase II. In addition, a systemic siRNA approach is under preclinical development against hypercholesterolemia. Other companies developing siRNA therapies are Nastech Pharmaceutical Company Inc. developing antiviral siRNAs against pandemic influenza. The Sirna Therapeutic is developing siRNAs targeting the VEGF for the

treatment of macular degeneration and hepatitis C virus. Protiva Biotherapeutics, is developing a lipid based siRNA delivery to target Ebola virus.

1.2.11 Combinatorial RNAi (coRNAi)

Numerous preclinical studies have shown the potency of RNA mediated silencing. However, common to all these mono-therapies is the moderate activity and, especially when used against viruses, escape of gene silencing by mutations or RNAi suppression. Escape mutations have been reported for polio, HIV and hepatitis [229-231]. In every replication cycle viruses such as HCV and HIV carry out multiple of mutations, making therapies based on high sequence homology inefficient. On the other hand, the RNAi suppression concept is suggested to happen via virally encoded suppressors of RNAi silencing. Recently, significant progress has been achieved by combining multiple gene silencing strategies. The method called combinatorial RNAi (coRNAi) can be divided into four fundamental approaches: 1) Combination of multiple siRNA against the different regions of the target gene, 2) inhibiting different target genes simultaneously e.g. in antiviral therapy targeting viral and cellular proteins and 3) co-expression with small RNAs and other RNA-based inhibitors 4) co-expressing small RNAs and proteins [232].

Today there are a number of examples suggesting the potency of coRNAi approaches. For example, many applications for expressing multiple shRNAs are effective for miRNA expression vectors. The use of miRNA vectors in this approach is well argued because of their use of the Pol II promoter, which allows more convenient concatamerization of hairpin under same promoter [233]. Song *et al.* have reported that co-expressing shRNA against cellular CCR5 receptor and viral p24 protein caused synergistic effects and almost complete inhibition of HIV infection [234]. However, even if this may not be the case for CCR5, there is risk of secondary effects when down regulating cellular genes. Jarczak *et al.*. showed the potential of shRNA in combination with hammer head ribozyme expression. The ribozymes increased shRNA effect considerably, indicating the advantage of co-expression of small RNAs and other RNA-based inhibitors [235]. Unwalla *et al.* took advantage of the common weakness of Pol II promoters using a minimal polyadenylation signal sequence to terminate the shRNA transcripts. They exploited the transcriptional read-through of a weak poly(A) signal as a means for coexpressing an shRNA and the antiviral transdominant RevM10 protein from the same promoter. By this they showed increased down regulation of HIV-1 gene expression [236]. The off-target effects can be utilized in some cases. Hornung *et al.* have shown that introduction of immunostimulatory sequences into sense strand of siRNA will awoke interferone-α response [237]. These immunostimulatory RNAs (isRNAs) could also serve as a possible application for futures development of coRNAi.

In the future, coRNAi seems to be the most promising alternative chasing the optimal small RNA-mediated approach. However, finding only one universal and omnipotent system is not likely. The specialized tailor made methods for each application and therapy will be the most probable choice in the future.

2 AIMS OF THE THESIS

Since 1993, when the Btk gene was cloned and associated to XLA, much effort has been directed to understanding the role of the Btk and other Tec family kinases. By developing and utilizing the latest technologies involving RNAi and microarray gene expression profiling, it has been possible to produce the novel information presented in this thesis.

- The fundamental aim was to discover new signaling molecules and cellular processes regulated by Btk.

- To utilize and develop RNAi as a tool for studying signaling in haematopoietic, difficult-to-transfect cells using RNAi: siRNA, shRNA, and inducible shRNA.

- To determine new candidates of Btk signaling by studying removal of Btk in haematopoietic cells including macrophages, B -, mast -and promonocytic cells.

3 ETHICS

All experiments were performed in accordance with good laboratory practice and with approved ethical permission (Paper IV) Dnr: S-94-03.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Tyrosine phosphorylations and membrane translocation of Btk take place upon activation of mast cells by antigen/IgE-mediated cross-linking of the FcεRI receptor [74,238-240]. In mast cells, Btk mutations lead to impairment in pro-inflammatory cytokine production [78], intracellular calcium influx and granule exocytosis [81], induced by cross-linking of high-affinity IgE receptor. Simultaneously, a vast number of signaling molecules have been reported to be involved in the signal transduction of Tec family kinases [9,241]. In this paper we have shown, that Btk can be silenced in hematopoietic cells by introducing short interfering RNAs (siRNA). This proof-of-concept silencing was achieved with conventional lipid-based transfection method and verified with immunoblotting. At that time the siRNA transfection efficiency was difficult to monitor. Therefore the siRNAs functionality in target cells was complemented with an alternative delivery method. SiRNAs were microinjected into target cells and the down regulation was monitored with fluorescent microscopy. Today, siRNA can be conjugated to a fluorescent dye, which makes it easier to follow the cellular uptake with microscopy or flow cytometry.

To obtain the best down regulation effect of the target gene we tested multiple siRNAs targeted to different areas of the coding region. Surprisingly, when combining 3 moderately good working siRNAs in the same transfection mixture, still having the same total amount of siRNA, the down-regulation of the target gene was shown to be more efficient (Fig. 7). According to our knowledge, this was the first time the beneficial effect of combinatorial siRNAs were shown.

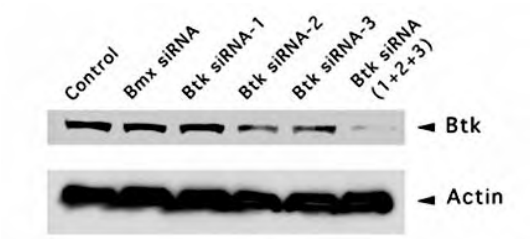


***Figure** 7. siRNA-mediated combinatorial inhibition of endogenous Btk expression in rat mast cells. siRNAs were designed against three different sites (1-3) in the Btk coding region. The expression of Btk in RBL-2H3 cells was analyzed 48 h post-transfection of siRNA duplexes 1-3 added separately or in triplet combination (final concentration 100 nM). The control lane was non-transfected cells and Bmx siRNA was irrelevant control-siRNA for Btk expression. The Btk expression was normalized to β-actin levels of total cell extracts.*

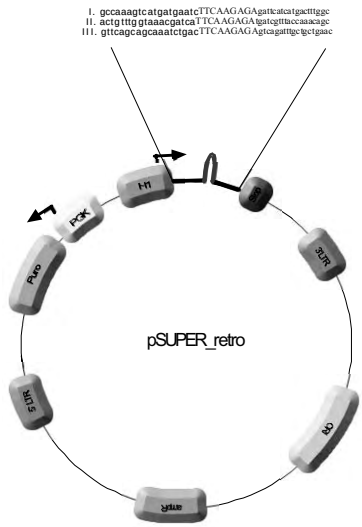
Furthermore, when analyzing the effects of Btk down regulation in the 2H3-RBL mast cell line, the histamine secretion was found to be decreased compared to wt. The same was observed when cells were treated with LFM-A13, a chemical inhibitor of Btk kinase activity. These experiments were done shortly after siRNA was proven to work

in mammalian cells. The data showed here provide evidence that partly explains functional mechanism of siRNA, and partly describes Btk's role in mast cell signaling.

4.2 PAPER II

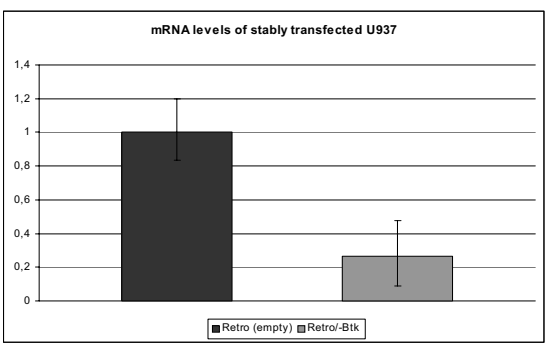
To achieve longer lasting silencing of the target gene short hairpin RNA (shRNA) expressing plasmids were utilized. ShRNA-oligos against Btk were inserted into a pSUPER_Retro vector and transfected with nucleofection into hematopoietic cells (Fig. 8).

a.



b.

c.



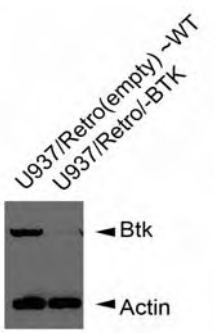


Figure 8. *Human monocytic cell line U937 was stably nucleofected with pSuper_retro and the down regulation capacity was measured with qRT-PCR and western blot. A. pSUPER/retro where the shRNA's against Btk were inserted to BglII and HindIII sites. B. Btk mRNA levels measured with qRT-PCR from pSuper_retro stable transfectants c. Western Blot analysis of Btk levels.*

The stable knock-down of Btk with 3 shRNA expressing plasmids was shown to be maintained in the target cells for several months. Frequent freeze-thawing did not affect the knock down level. Combining multiple shRNA-expressing plasmids against the

target gene has become a state-of-the-art method used in shRNA-based approaches. Moreover, it has been shown that by co-expressing irrelevant miRNA sequences in combination with a relevant sequence, it is possible to increase the efficacy of the single functional hairpin [232]. The mechanism of action is not known. However, this supports our earlier finding, where adding multiple siRNA against the same target mRNA will increase knock down of the target gene (Paper I and [242]).

Recently, it has been reported by several different groups that high copy numbers of shRNA plasmids will decrease the RNAi effect. The system is not completely understood, but one possible mechanism might be the saturation of the RNAi machinery. This is a possible draw back of our approach where nucleofection of different shRNA caused heterogeneous shRNA expressing population. By starting the selection of shRNA expressing cell from single clones, it would be possible to monitor more accurately the number of shRNA insertions and thereby the expression levels of shRNAs. The U6 promoter has been shown to be transcriptionally stronger compared to H1. However, lower amounts of shRNA transcripts produced by the H1 promoter has been proposed to be more advantageous for longtime stable expression [232,243].

The Btk gene expression was efficiently down regulated in U937 promonocytic cells while no differences was observed in mouse B cell line A20. The down regulation of Btk in Raji resulted in cell death monitored by apoptosis assay. The result was surprising since Raji B cell lymphoma cells are characterized as mature B cells and the Btk's role in mature B cells has not been reported to be crucial for viability. Btk's role was further analyzed in promonocytes and PMA differentiated macrophages. The results show that Btk is more important to macrophage than monocyte signaling. This was assayed with gene expression profiling, which showed 11 Btk regulated transcripts in promonocytes and 58 in PMA differentiated macrophages (fold change criteria >2.0). Clustering analysis was used for further study by arranging the hits by their presence in different comparison groups. By comparing wt_ss/wt_PMA to Btk_kd_ss/Btk_kd_PMA 349 fewer transcripts were found in PMA stimulated Btk knock-down cells compared to parental PMA stimulated. Taking into account wt_ss/Btk_kd_ss and wt_PMA/Btk_kd_PMA comparisons we could identify 5 Btk-dependent transcripts that were only detected in PMA stimulated U937 cells. These transcripts; CD89/FcαR (FCAR), Resistin, GABRA2, β1 integrin and GULP1 correspond to genes that have been suggested earlier to play a role in macrophage effector functions [63] and are now shown to be regulated by Btk. Further studies need to be done in order to verify our discovery. In addition, in this manuscript we show that combining an shRNA approach with microarray analysis is a powerful tool for signaling research. Moreover, the data presented here confirms that Btk expression can regulate many important genes in macrophage signaling.

4.3 PAPER III

siRNAs can, as previously described, be introduced into cells with shRNA-plasmids. shRNA plasmids, sometimes referred to as second-generation of siRNA technology, enabled the creation of stably transfected cell lines, leading under optimal conditions to a sustained knockdown of the target gene by ~90% of its expression. However, the technique was not suitable for studying genes involved in maintaining survival or for studying immediate responses following down regulation. One example of that type of situation was faced in paper II when down regulation of Btk in Raji cells resulted in cell death. To overcome this restriction, a lentiviral inducible shRNA-producing plasmid pLentiINDucible (pLIND) was designed. These third-generation siRNAs were first created with tetracycline-based methods [168,190] and, more recently, as in this paper using the CRE-LoxP system [199-202]. None of these vector systems had the optimal combination of elements and functions for tightly regulated and efficient induction of shRNA in both mouse and human cells. However, the approach used here has many advantages compared to its challengers. The pLIND system uses the human H1 promoter, is selectable against enhanced green fluorescent protein (EGFP), and can be induced by a nlsCRE/IRES2/DsRed2 plasmid (Fig.9-11). The vector design shows the power and the importance of the placement of the promoter, LoxP, and the siRNA-producing cassette (Fig. 10). The strength of this system is its robust hairpin production, selection ability against EGFP, and specificity compared with earlier tetracycline-induced systems [244]. Furthermore, the human H1 promoter makes it possible to use this method in both human and mouse cells, whereas mouse U6 promoter-driven vectors are restricted to mouse cells [245]. The disadvantages of this approach are its irreversibility, the need for a second inducible plasmid for Cre and the loss of the selection marker after induction.

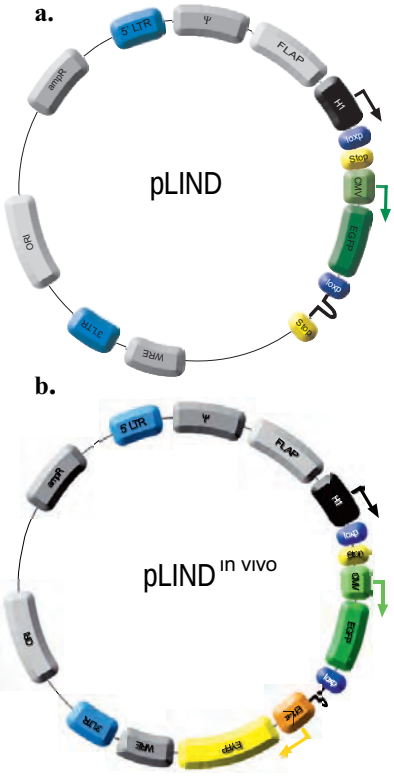


Figure 9. *Lentiviral inducible shRNA vectors a) pLIND and b) pLIND$^{in\ vivo}$. Vectors share the similar backbones; they differ in additional selection marker cassette which was added into pLIND$^{in\ vivo}$ (Unpublished results).*

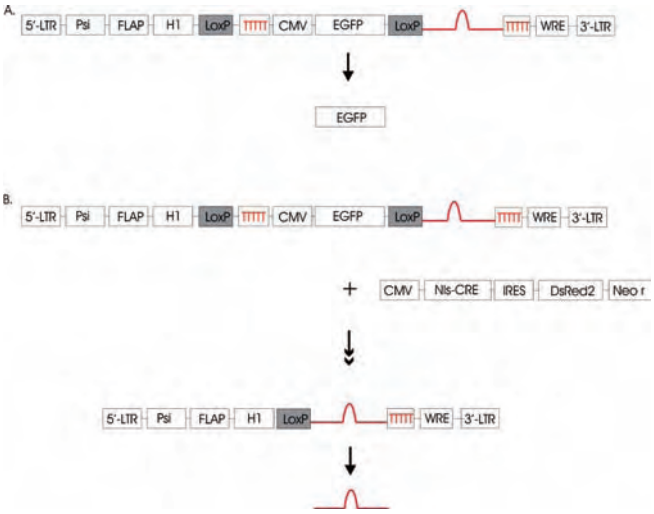


Figure 10. *Schematic representation of inducible pLIND vector system. **(A)** The pLIND plasmid alone allows the EGFP stuffer reporter cassette to be expressed under the CMV promoter. The shRNA remains unexpressed, as the H1 promoter is not functional due to the presence of a termination sequence (five thymidines) after the first LoxP site and two clusters of four repetitive thymidines inside the CMV promoter. **(B)** After the expression of pNLSCRE/IRES2/DsRed2 plasmid, the CRE will cause LoxP recombination, which will remove the EGFP stuffer cassette, and the shRNA will be expressed. [LTR, long terminal repeat; Psi, extended viral packaging signal; FLAP, HIV-1 flap element; WRE, woodchuck hepatitis virus posttranscriptional regulatory element; EGFP, enhanced green fluorescent protein; IRES, internal ribosome entry site; DsRed2, Discosoma sp. red fluorescent protein 2.]*

To overcome these limitations we have recently developed a new variant of pLIND vector; pLIND$^{\text{in vivo}}$. This vector contains an additional selection cassette for active (Ef-1α/EYFP) shRNA production (Fig. 9b). The functionality of the vector has been tested in primary cells and appears to have potential for *in vivo* trials (Heinonen *et al.* unpublished results). We have also developed an inducible variant of a Cre expression vector which is now under evaluation (Heinonen *et al.* unpublished results). The inducible siRNA system will be a valuable tool for vast number of applications.

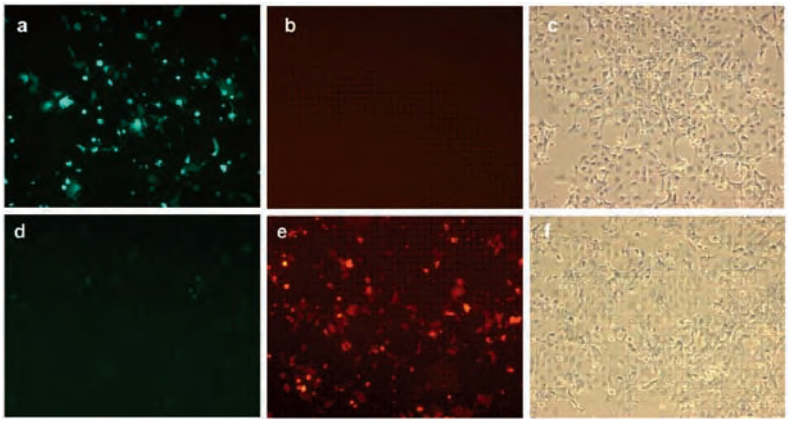


Figure 10. *Fluorescence microscope monitoring of Cre-inducible shRNA production, followed by EGFP stuffer reporter deletion. Transfection of Cos-7 cells with pLIND alone **(a, b, c)** or together with pNLS Cre/IRES2/DsRed2 **(d, e, f)**. Images were taken under FITC-filter **(a, d)**, Cy-3 filter **(b, e)**, and brightfield **(c, f)**.*

4.4 PAPER IV

In this work the role of Btk in bone marrow-derived mast cells (BMMC) was addressed. The cells were extracted from CBA and C57BL/6J mice and cultivated under IL-3 and SCF. By using Btk protein level as an indicator we studied BMMC growth by mixing 19/20 parts of Btk deficient cells and 1/20 part wt BMMC. After three weeks of cultivation the Btk protein level in this heterogenous population was comparable to wt BMMC, stating that almost all the cells in culture were wt BMMC. Moreover, this suggests that Btk-containing cells divide faster compared to Btk-deficient. The cell cycle analysis revealed the reason for this; Btk deficient cells were prone to G2/M arrest compared to wt cells. Our data suggest that a similar G2 arrest as seen in STAT5-/- BMMC also exists in Btk-/- BMMC. Btk-mediated STAT5 activation has been shown to occur via cytokine- as well as antigen receptor mediated signaling [55,246]. Cell death was increased in wt compared to Btk-deficient BMMC. These results reflects the fact that wt BMMC have more frequent turn-over compared Btk-deficient cells that are arrested in G2 phase.

To further dissect the Btk signaling in BMMC microarray analysis was performed. After analyzing triplicate samples with fold-change criteria of ≥ 2.0, we found transcripts identified by 50 probe sets to be affected by Btk removal. Out of those, 21 were statistically significant ($p \leq 0.05$) representing 15 different genes. Of the remaining Tec family members none was found to be differentially expressed following removal of Btk.

Interestingly, CREB1 was up-regulated more than 3-fold in Btk-/- BMMC. Recently, CREB1 has been implicated to play a role in myeloid cell transformation [247,248]. CREB1 induces cyclin A1 expression, associated also with hematopoietic malignancies, and explains enhanced the proliferation and increased numbers of cells in S phase. Since CREB1 was up-regulated in Btk-/- mast cells, this could also be one possible mechanism for the increased survival of Btk deficient BMMC. Another transcript identified with 3 different probe sets representing the *Mela* gene was up-regulated on average 60-fold in Btk -/- BMMC. This was also confirmed with whole splenic B cells from C57BL6 mice and transitional type 1 B cells from CBA mice, where the up-regulation followed same tendency, but with less fold-change. No expression of Mela was found in wt BMMC or mouse B cells. Reduced, or aberrant, expression of Btk has been shown to increase pre-B cell tumor incidence in mouse models [88]. This finding supports the speculations of Btk's role tumor suppressor in cancer. From the 21 probe sets identifying transcripts regulated by Btk, three corresponds to a novel G-coupled protein receptor 177 (GPR177). GPR177 expression was up-regulated on average 5-fold in $Btk^{-/-}$ BMMC which suggests its negative regulation by Btk. A number of reports have described Btk's direct interaction with heterotrimeric G-protein $\beta\gamma$ subunits as well as $G\alpha q$ and $G\alpha 12$ subunits [84,249,250]. However, the receptors related to these interactions have not been fully determined. GPR177 was also found up-regulated from Btk deficient transitional type 1 B cells. GPR177 is located on chromosome 1p31 in humans, the same area which

was recently found to be specifically linked to the co-morbidity of asthma and allergic rhinitis [251].

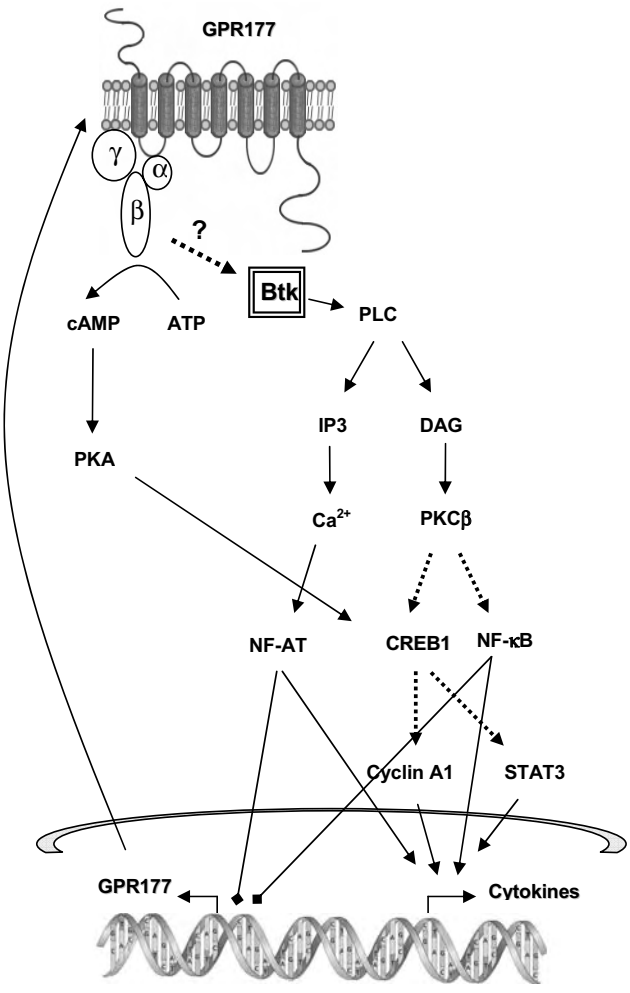


Figure 12. *A working model for GPR177 and Btk signaling in BMMC.*

Defective Btk signaling has been shown to affect serum IgE concentration and many cytokine expression e.g. IL-4, IL-5, IL-13 and IFN-γ [246]. Many of these genes are asthma candidate genes. Moreover, a new orphan G protein-coupled receptor GPRA/GPR154 has been related to the pathogenesis of atopy and asthma [252]. In spite of that, evidences suggests a possible link between Btk and allergic disorders, no direct evidence has been presented nor found. Moreover, the implication and relationship of GPR177 and Btk needs to be further determined in protein levels as well as in human samples. The model in Figure 12 proposes Btk mediated regulation of GPR177 in mast cells.

5 CLOSING REMARKS

Btk signaling has been studied by developing and utilizing siRNA technology followed by gene expression profiling. The studies were done in hematopoietic cells including monocytes, mast and B cells, which are the natural environment for Btk expression and function. What has then been achieved? The multiple siRNA approach has been shown to be beneficial in efficient target gene knock down (Paper I). Furthermore, the evidence supporting Btk's role in allergy was presented. Novel Btk regulated receptor GPR177 was identified providing a possible link for GPCR and Btk signaling in mast cells (Paper IV). The evidence provided here supports Btk's role as a tumor suppressor in mast and B cells. This statement is based on the discovery of a robust up-regulation MeLa in Btk deficient cells (Paper IV). The proof-of-principle for using the stable shRNA mediated gene knock down in combination with gene expression profiling in cell signaling research was presented (Paper II). Moreover, evidence supporting Btk's role in macrophage effector function signaling has been presented. The inducible siRNA vector system was developed, providing a convenient tool for efficient target gene knockdown cells that are difficult to transfect (paper III). In light of these new results we have taken one step forward in describing of the fundamental role of Btk in hematoipoietic cells.

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7 REFERENCES

[1] Tsukada, S. et al. (1993). Deficient expression of a B cell cytoplasmic tyrosine kinase in human X-linked agammaglobulinemia. Cell 72, 279-90.

[2] Vetrie, D. et al. (1993). The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature 361, 226-233.

[3] Rawlings, D.J. et al. (1993). Mutation of unique region of Bruton's tyrosine kinase in immunodeficient XID mice. Science 261, 358-61.

[4] Sideras, P. et al. (1994). Genomic organization of mouse and human Bruton's agammaglobulinemia tyrosine kinase (Btk) loci. J Immunol 153, 5607-5617.

[5] Smith, C.I. and Notarangelo, L.D. (1997). Molecular basis for X-linked immunodeficiencies. Adv Genet 35, 57-115.

[6] Himmelmann, A., Thevenin, C., Harrison, K. and Kehrl, J.H. (1996). Analysis of the Bruton's tyrosine kinase gene promoter reveals critical PU.1 and SP1 sites. Blood 87, 1036-1044.

[7] Muller, S., Sideras, P., Smith, C.I. and Xanthopoulos, K.G. (1996). Cell specific expression of human Bruton's agammaglobulinemia tyrosine kinase gene (Btk) is regulated by Sp1- and Spi-1/PU.1-family members. Oncogene 13, 1955-64.

[8] Brunner, C. and Wirth, T. (2006). Btk expression is controlled by Oct and BOB.1/OBF.1. Nucleic Acids Res 34, 1807-1815.

[9] Smith, C.I., Islam, T.C., Mattsson, P.T., Mohamed, A.J., Nore, B.F. and Vihinen, M. (2001). The Tec family of cytoplasmic tyrosine kinases: mammalian Btk, Bmx, Itk, Tec, Txk and homologs in other species. Bioessays 23, 436-46.

[10] Yu, H., Chen, J.K., Feng, S., Dalgarno, D.C., Brauer, A.W. and Schreiber, S.L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell 76, 933-45.

[11] Hidaka, M., Homma, Y. and Takenawa, T. (1991). Highly conserved eight amino acid sequence in SH2 is important for recognition of phosphotyrosine site. Biochem Biophys Res Commun 180, 1490-1497.

[12] Mahajan, S., Fargnoli, J., Burkhardt, A.L., Kut, S.A., Saouaf, S.J. and Bolen, J.B. (1995). Src family protein tyrosine kinases induce autoactivation of Bruton's tyrosine kinase. Mol Cell Biol 15, 5304-5311.

[13] Mao, C., Zhou, M. and Uckun, F.M. (2001). Crystal Structure of Bruton's Tyrosine Kinase Domain Suggests a Novel Pathway for Activation and Provides Insights into the Molecular Basis of X-linked Agammaglobulinemia. J Biol Chem 276, 41435-41443.

[14] Campana, D., Farrant, J., Inamdar, N., Webster, A.D. and Janossy, G. (1990). Phenotypic features and proliferative activity of B cell progenitors in X-linked agammaglobulinemia. J Immunol 145, 1675-1680.

[15] Thomas, J.D., Sideras, P., Smith, C.I., Vorechovsky, I., Chapman, V. and Paul, W.E. (1993). Colocalization of X-linked agammaglobulinemia and X-linked immunodeficiency genes. Science 261, 355-8.

[16] Rigley, K.P., Harnett, M.M., Phillips, R.J. and Klaus, G.G. (1989). Analysis of signaling via surface immunoglobulin receptors on B cells from CBA/N mice. Eur J Immunol 19, 2081-6.

[17] Wicker, L.S. and Scher, I. (1986). X-linked immune deficiency (xid) of CBA/N mice. Curr Top Microbiol Immunol 124, 87-101.
[18] Kerner, J.D., Appleby, M.W., Mohr, R.N., Chien, S., Rawlings, D.J., Maliszewski, C.R., Witte, O.N. and Perlmutter, R.M. (1995). Impaired expansion of mouse B cell progenitors lacking Btk. Immunity 3, 301-12.
[19] Khan, W.N. et al. (1995). Defective B cell development and function in Btk-deficient mice. Immunity 3, 283-99.
[20] Vihinen, M. et al. (1998). BTKbase, mutation database for X-linked agammaglobulinemia (XLA). Nucleic Acids Res 26, 242-247.
[21] Wang, D. et al. (2000). Phospholipase C[gamma]2 Is Essential in the Functions of B Cell and Several Fc Receptors. Immunity 13, 25-35.
[22] Fruman, D.A., Snapper, S.B., Yballe, C.M., Davidson, L., Yu, J.Y., Alt, F.W. and Cantley, L.C. (1999). Impaired B Cell Development and Proliferation in Absence of Phosphoinositide 3-Kinase p85. Science 283, 393-397.
[23] Jumaa, H., Wollscheid, B., Mitterer, M., Wienands, J., Reth, M. and Nielsen, P.J. (1999). Abnormal Development and Function of B Lymphocytes in Mice Deficient for the Signaling Adaptor Protein SLP-65. Immunity 11, 547-554.
[24] Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S. and Tarakhovsky, A. (1996). Immunodeficiency in protein kinase cbeta-deficient mice. Science 273, 788-91.
[25] Lindvall, J.M. et al. (2005). Bruton's tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. Immunol Rev 203, 200-215.
[26] Mohamed, A.J., Vargas, L., Nore, B.F., Backesjo, C.-M., Christensson, B. and Smith, C.I.E. (2000). Nucleocytoplasmic Shuttling of Bruton's Tyrosine Kinase. J Biol Chem 275, 40614-40619.
[27] Brunner, C., Muller, B. and Wirth, T. (2005). Bruton's Tyrosine Kinase is involved in innate and adaptive immunity. Histol Histopathol 20, 945-55.
[28] Klaus, G.G., Holman, M., Johnson-Leger, C., Elgueta-Karstegl, C. and Atkins, C. (1997). A re-evaluation of the effects of X-linked immunodeficiency (xid) mutation on B cell differentiation and function in the mouse. Eur J Immunol 27, 2749-56.
[29] Ogata, H. et al. (2000). The Toll-like Receptor Protein RP105 Regulates Lipopolysaccharide Signaling in B Cells. J Exp Med 192, 23-30.
[30] Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. and Akira, S. (1999). Cutting Edge: Toll-Like Receptor 4 (TLR4)-Deficient Mice Are Hyporesponsive to Lipopolysaccharide: Evidence for TLR4 as the Lps Gene Product. J Immunol 162, 3749-3752.
[31] Spaargaren, M., Beuling, E.A., Rurup, M.L., Meijer, H.P., Klok, M.D., Middendorp, S., Hendriks, R.W. and Pals, S.T. (2003). The B Cell Antigen Receptor Controls Integrin Activity through Btk and PLC{gamma}2. J Exp Med 198, 1539-1550.
[32] Antony, P., Petro, J.B., Carlesso, G., Shinners, N.P., Lowe, J. and Khan, W.N. (2004). B-cell antigen receptor activates transcription factors NFAT (nuclear factor of activated T-cells) and NF-kappaB (nuclear factor kappaB) via a mechanism that involves diacylglycerol. Biochem Soc Trans 32, 113-5.

[33] Hao, S., Kurosaki, T. and August, A. (2003). Differential regulation of NFAT and SRF by the B cell receptor via a PLCgamma-Ca(2+)-dependent pathway. Embo J 22, 4166-77.

[34] Jiang, A., Craxton, A., Kurosaki, T. and Clark, E.A. (1998). Different Protein Tyrosine Kinases Are Required for B Cell Antigen Receptor-mediated Activation of Extracellular Signal-Regulated kinase, c-Jun NH2-terminal Kinase 1, and p38 Mitogen-activated Protein Kinase. J Exp Med 188, 1297-1306.

[35] Lindvall, J. and Islam, T.C. (2002). Interaction of Btk and Akt in B cell signaling. Biochem Biophys Res Commun 293, 1319-1326.

[36] Petro, J.B., Rahman, S.M.J., Ballard, D.W. and Khan, W.N. (2000). Bruton's Tyrosine Kinase Is Required for Activation of I{kappa}B Kinase and Nuclear Factor {kappa}B in Response to B Cell Receptor Engagement. J Exp Med 191, 1745-1754.

[37] Petro, J.B., Castro, I., Lowe, J. and Khan, W.N. (2002). Bruton's tyrosine kinase targets NF-[kappa]B to the bcl-x promoter via a mechanism involving phospholipase C-[gamma]2 following B cell antigen receptor engagement. FEBS Letters 532, 57-60.

[38] Saijo, K., Mecklenbrauker, I., Santana, A., Leitger, M., Schmedt, C. and Tarakhovsky, A. (2002). Protein Kinase C {beta} Controls Nuclear Factor {kappa}B Activation in B Cells Through Selective Regulation of the I{kappa}B Kinase {alpha}. J Exp Med 195, 1647-1652.

[39] Craxton, A., Jiang, A., Kurosaki, T. and Clark, E.A. (1999). Syk and Bruton's Tyrosine Kinase Are Required for B Cell Antigen Receptor-mediated Activation of the Kinase Akt. J Biol Chem 274, 30644-30650.

[40] Saito, K. et al. (2003). BTK Regulates PtdIns-4,5-P2 Synthesis: Importance for Calcium Signaling and PI3K Activity. Immunity 19, 669-677.

[41] Matsushita, M. et al. (1998). Identification and Characterization of a Novel SH3-Domain Binding Protein, Sab, Which Preferentially Associates with Bruton's Tyrosine Kinase (Btk). Biochemical and Biophysical Research Communications 245, 337-343.

[42] Liu, W., Quinto, I., Chen, X., Palmieri, C., Rabin, R.L., Schwartz, O.M., Nelson, D.L. and Scala, G. (2001). Direct inhibition of Bruton's tyrosine kinase by IBtk, a Btk-binding protein. Nat Immunol 2, 939-946.

[43] Kang, S.W. et al. (2001). PKCbeta modulates antigen receptor signaling via regulation of Btk membrane localization. Embo J 20, 5692-702.

[44] Bolland, S., Pearse, R.N., Kurosaki, T. and Ravetch, J.V. (1998). SHIP Modulates Immune Receptor Responses by Regulating Membrane Association of Btk. Immunity 8, 509-516.

[45] Yu, L., Mohamed, A.J., Vargas, L., Berglof, A., Finn, G., Lu, K.P. and Smith, C.I.E. (2006). Regulation of Bruton Tyrosine Kinase by the Peptidylprolyl Isomerase Pin1. J Biol Chem 281, 18201-18207.

[46] Vassilev, A., Ozer, Z., Navara, C., Mahajan, S. and Uckun, F.M. (1999). Bruton's tyrosine kinase as an inhibitor of the Fas/CD95 death-inducing signaling complex. J Biol Chem 274, 1646-56.

[47] Uckun, F.M., Waddick, K.G., Mahajan, S., Jun, X., Takata, M., Bolen, J. and Kurosaki, T. (1996). BTK as a mediator of radiation-induced apoptosis in DT-40 lymphoma B cells. Science 273, 1096-100.

[48] Kawakami, Y. et al. (1997). Bruton's tyrosine kinase regulates apoptosis and JNK/SAPK kinase activity. Proc Natl Acad Sci U S A 94, 3938-3942.

[49] Forssell, Nilsson and Sideras. (1999). Bruton's Tyrosine-Kinase-Deficient Murine B Lymphocytes Fail to Enter S Phase when Stimulated with Anti-Immunoglobulin Plus Interleukin-4. Scand J Immunol 49, 155-161.

[50] Solvason, N. et al. (1998). Transgene Expression of bcl-xL Permits Anti-immunoglobulin (Ig)-induced Proliferation in xid B Cells. J Exp Med 187, 1081-1091.

[51] Glassford, J. et al. (2003). BCR targets cyclin D2 via Btk and the p85alpha subunit of PI3-K to induce cell cycle progression in primary mouse B cells. Oncogene 22, 2248-59.

[52] Egloff, A.M. and Desiderio, S. (2001). Identification of Phosphorylation Sites for Bruton's Tyrosine Kinase within the Transcriptional Regulator BAP/TFII-I. J Biol Chem 276, 27806-27815.

[53] Novina, C.D., Kumar, S., Bajpai, U., Cheriyath, V., Zhang, K., Pillai, S., Wortis, H.H. and Roy, A.L. (1999). Regulation of Nuclear Localization and Transcriptional Activity of TFII-I by Bruton's Tyrosine Kinase. Mol Cell Biol 19, 5014-5024.

[54] Hirano, M., Kikuchi, Y., Nisitani, S., Yamaguchi, A., Satoh, A., Ito, T., Iba, H. and Takatsu, K. (2004). Bruton's tyrosine kinase (Btk) enhances transcriptional co-activation activity of BAM11, a Btk-associated molecule of a subunit of SWI/SNF complexes. Intern Immunol 16, 747-757.

[55] Mahajan, S., Vassilev, A., Sun, N., Ozer, Z., Mao, C. and Uckun, F.M. (2001). Transcription factor STAT5A is a substrate of Bruton's tyrosine kinase in B cells. J Biol Chem 276, 31216-28.

[56] Martino, A., Holmes, J.H.I.V., Lord, J.D., Moon, J.J. and Nelson, B.H. (2001). Stat5 and Sp1 Regulate Transcription of the Cyclin D2 Gene in Response to IL-2. J Immunol 166, 1723-1729.

[57] Horwood, N.J. et al. (2006) Bruton's Tyrosine Kinase Is Required for TLR2 and TLR4-Induced TNF, but Not IL-6, Production,. J Immunol 176, 3635-3641

[58] Gray, P., Dunne, A., Brikos, C., Jefferies, C.A., Doyle, S.L. and O'Neill, L.A.J. (2006). MyD88 Adapter-like (Mal) Is Phosphorylated by Bruton's Tyrosine Kinase during TLR2 and TLR4 Signal Transduction. J Biol Chem 281, 10489-10495.

[59] Jefferies, C.A. et al. (2003). Bruton's Tyrosine Kinase Is a Toll/Interleukin-1 Receptor Domain-binding Protein That Participates in Nuclear Factor {kappa}B Activation by Toll-like Receptor 4. J Biol Chem 278, 26258-26264.

[60] Schmidt, N.W., Thieu, V.T., Mann, B.A., Ahyi, A.-N.N. and Kaplan, M.H. (2006). Bruton's Tyrosine Kinase Is Required for TLR-Induced IL-10 Production. J Immunol 177, 7203-7210.

[61] Horwood, N.J. et al. (2006). Bruton's Tyrosine Kinase Is Required for TLR2 and TLR4-Induced TNF, but Not IL-6, Production. J Immunol 176, 3635-3641.

[62] Mukhopadhyay, S., George, A., Bal, V., Ravindran, B. and Rath, S. (1999). Bruton's Tyrosine Kinase Deficiency in Macrophages Inhibits Nitric Oxide Generation Leading to Enhancement of IL-12 Induction. J Immunol 163, 1786-1792.

[63] Mukhopadhyay, S., Mohanty, M., Mangla, A., George, A., Bal, V., Rath, S. and Ravindran, B. (2002). Macrophage Effector Functions Controlled by Bruton's

Tyrosine Kinase Are More Crucial Than the Cytokine Balance of T Cell Responses for Microfilarial Clearance. J Immunol 168, 2914-2921.

[64] Amoras, A.L., Kanegane, H., Miyawaki, T. and Vilela, M.M. (2003). Defective Fc-, CR1- and CR3-mediated monocyte phagocytosis and chemotaxis in common variable immunodeficiency and X-linked agammaglobulinemia patients. J Investig Allergol Clin Immunol 13, 181-8.

[65] Reyes-Reyes, M., Mora, N., Gonzalez, G. and Rosales, C. (2002). beta1 and beta2 integrins activate different signalling pathways in monocytes. Biochem J 363, 273-280.

[66] Reyes-Reyes, M., Mora, N., Zentella, A. and Rosales, C. (2001). Phosphatidylinositol 3-kinase mediates integrin-dependent NF-(&kgr;)B and MAPK activation through separate signaling pathways. J Cell Sci 114, 1579-1589.

[67] Rosales, C. and Juliano, R. (1996). Integrin signaling to NF-kappa B in monocytic leukemia cells is blocked by activated oncogenes. Cancer Res 56, 2302-5.

[68] Perez de Diego, R. et al. (2006). Bruton's tyrosine kinase is not essential for LPS-induced activation of human monocytes. Journal of Allergy and Clinical Immunology 117, 1462-1469.

[69] Launay, P., Lehuen, A., Kawakami, T., Blank, U. and Monteiro, R.C. (1998). IgA Fc receptor (CD89) activation enables coupling to syk and Btk tyrosine kinase pathways: differential signaling after IFN-gamma or phorbol ester stimulation. J Leuk Biol 63, 636-642.

[70] Lang, M.L., Chen, Y.W., Shen, L., Gao, H., Lang, G.A., Wade, T.K. and Wade, W.F. (2002). IgA Fc receptor (FcalphaR) cross-linking recruits tyrosine kinases, phosphoinositide kinases and serine/threonine kinases to glycolipid rafts. Biochem J 364, 517-25.

[71] Vargas, L., Nore, B.F., Berglof, A., Heinonen, J.E., Mattsson, P.T., Smith, C.I.E. and Mohamed, A.J. (2002). Functional Interaction of Caveolin-1 with Bruton's Tyrosine Kinase and Bmx. J Biol Chem 277, 9351-9357.

[72] Shen, L. et al. (2001). Presentation of ovalbumin internalized via the immunoglobulin-A Fc receptor is enhanced through Fc receptor {gamma}-chain signaling. Blood 97, 205-213.

[73] Metz, M. and Maurer, M. (2007). Mast cells - key effector cells in immune responses. Trends in Immunology 28, 234-241.

[74] Kawakami, Y., Kitaura, J., Hartman, S.E., Lowell, C.A., Siraganian, R.P. and Kawakami, T. (2000). Regulation of protein kinase CbetaI by two protein-tyrosine kinases, Btk and Syk. Proc Natl Acad Sci U S A 97, 7423-8.

[75] Iwaki, S., Tkaczyk, C., Satterthwaite, A.B., Halcomb, K., Beaven, M.A., Metcalfe, D.D. and Gilfillan, A.M. (2005). Btk Plays a Crucial Role in the Amplification of Fc{epsilon}RI-mediated Mast Cell Activation by Kit. J Biol Chem 280, 40261-40270.

[76] Kitaura, J., Asai, K., Maeda-Yamamoto, M., Kawakami, Y., Kikkawa, U. and Kawakami, T. (2000). Akt-dependent Cytokine Production in Mast Cells. J Exp Med 192, 729-740.

[77] Hata, D., Kitaura, J., Hartman, S.E., Kawakami, Y., Yokota, T. and Kawakami, T. (1998). Bruton's Tyrosine Kinase-mediated Interleukin-2 Gene Activation in

Mast Cells. Dependence on the c-Jun N-terminal kinase activation pathway. J Biol Chem 273, 10979-10987.

[78] Hata, D. et al. (1998). Involvement of Bruton's Tyrosine Kinase in Fcepsilon RI-dependent Mast Cell Degranulation and Cytokine Production. Exp. Med. 187, 1235-1247.

[79] Heinonen, J.E., Smith, C.I. and Nore, B.F. (2002). Silencing of Bruton's tyrosine kinase (Btk) using short interfering RNA duplexes (siRNA). FEBS Lett 527, 274-8.

[80] Kawakami, Y. et al. (2000). Redundant and opposing functions of two tyrosine kinases, Btk and Lyn, in mast cell activation. J Immunol 165, 1210-9.

[81] Setoguchi, R., Kinashi, T., Sagara, H., Hirosawa, K. and Takatsu, K. (1998). Defective degranulation and calcium mobilization of bone-marrow derived mast cells from Xid and Btk-deficient mice. Immunol Lett 64, 109-18.

[82] Bence, K., Ma, W., Kozasa, T. and Huang, X.-Y. (1997). Direct stimulation of Bruton's tyrosine kinase by Gq-protein [alpha]-subunit. Nature 389, 296-299.

[83] Jiang, Y., Ma, W., Wan, Y., Kozasa, T., Hattori, S. and Huang, X.-Y. (1998). The G protein G[alpha]12 stimulates Bruton's tyrosine kinase and a rasGAP through a conserved PH/BM domain. Nature 395, 808-813.

[84] Langhans-Rajasekaran, S.A., Wan, Y. and Huang, X.Y. (1995). Activation of Tsk and Btk tyrosine kinases by G protein beta gamma subunits. Proc Natl Acad Sci U S A 92, 8601-5.

[85] Lowry, W.E. and Huang, X.Y. (2002). G Protein beta gamma subunits act on the catalytic domain to stimulate Bruton's agammaglobulinemia tyrosine kinase. J Biol Chem 277, 1488-92.

[86] Grabiner, B.C. et al. (2007). CARMA3 deficiency abrogates G protein-coupled receptor-induced NF-{kappa}B activation. Genes Dev 21, 984-996.

[87] Wegener, E. and Krappmann, D. (2007). CARD-Bcl10-Malt1 Signalosomes: Missing Link to NF-{kappa}B. Sci STKE, 384, pe21-.

[88] Kersseboom, R., Middendorp, S., Dingjan, G.M., Dahlenborg, K., Reth, M., Jumaa, H. and Hendriks, R.W. (2003). Bruton's Tyrosine Kinase Cooperates with the B Cell Linker Protein SLP-65 as a Tumor Suppressor in Pre-B Cells. J Exp Med 198, 91-98.

[89] Middendorp, S., Zijlstra, A.J.E., Kersseboom, R., Dingjan, G.M., Jumaa, H. and Hendriks, R.W. (2005). Tumor suppressor function of Bruton tyrosine kinase is independent of its catalytic activity. Blood 105, 259-265.

[90] Goodman, P.A., Wood, C.M., Vassilev, A.O., Mao, C. and Uckun, F.M. (2003). Defective expression of Bruton's tyrosine kinase in acute lymphoblastic leukemia. Leuk Lymphoma 44, 1011-8.

[91] Li, T., Tsukada, S., Satterthwaite, A., Havlik, M.H., Park, H., Takatsu, K. and Witte, O.N. (1995). Activation of Bruton's tyrosine kinase (BTK) by a point mutation in its pleckstrin homology (PH) domain. Immunity 2, 451-60.

[92] Costa, F.F. (2007). Non-coding RNAs: Lost in translation? Gene 386, 1-10.

[93] Kapranov, P., Drenkow, J., Cheng, J., Long, J., Helt, G., Dike, S. and Gingeras, T.R. (2005). Examples of the complex architecture of the human transcriptome revealed by RACE and high-density tiling arrays. Genome Res 15, 987-997.

[94] Wagner, R.W. (1994). Gene inhibition using antisense oligodeoxynucleotides. Nature 372, 333-5.

[95] Ho, S.P., Bao, Y., Lesher, T., Malhotra, R., Ma, L.Y., Fluharty, S.J. and Sakai, R.R. (1998). Mapping of RNA accessible sites for antisense experiments with oligonucleotide libraries. Nat Biotechnol 16, 59-63.

[96] Paterson, B.M., Roberts, B.E. and Kuff, E.L. (1977). Structural gene identification and mapping by DNA-mRNA hybrid-arrested cell-free translation. Proc Natl Acad Sci U S A 74, 4370-4.

[97] Stephenson, M.L. and Zamecnik, P.C. (1978). Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc Natl Acad Sci U S A 75, 285-8.

[98] Zamecnik, P.C. and Stephenson, M.L. (1978). Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci U S A 75, 280-4.

[99] Stein, C.A. and Cheng, Y.C. (1993). Antisense oligonucleotides as therapeutic agents--is the bullet really magical? Science 261, 1004-12.

[100] Crooke, S.T. (1993). Progress toward oligonucleotide therapeutics: pharmacodynamic properties. Faseb J 7, 533-539.

[101] Devi, G.R. (2006). siRNA-based approaches in cancer therapy. Cancer Gene Ther 13, 819-29.

[102] Hoyne, P.R., Edwards, L.M., Viari, A. and Maher, L.J. (2000). Searching genomes for sequences with the potential to form intrastrand triple helices. Journal of Molecular Biology 302, 797-809.

[103] Frank-Kamenetskii, M.D. and Mirkin, S.M. (1995). Triplex DNA structures. Annu Rev Biochem 64, 65-95.

[104] Agrawal, S. and Zhao, Q. (1998). Mixed backbone oligonucleotides: improvement in oligonucleotide-induced toxicity in vivo. Antisense Nucleic Acid Drug Dev 8, 135-9.

[105] Ge, R., Heinonen, J.E., Svahn, M.G., Mohamed, A.J., Lundin, K.E. and Smith, C.I.E. (2007). Zorro locked nucleic acid induces sequence-specific gene silencing. Faseb J 21, 1902-1914.

[106] Miyagishi, M., Hayashi, M. and Taira, K. (2003). Comparison of the suppressive effects of antisense oligonucleotides and siRNAs directed against the same targets in mammalian cells. Antisense Nucleic Acid Drug Dev 13, 1-7.

[107] Wingard, S.A. (1928). Hosts and symptoms of ring spot, a virus disease of plants. J. Agric. Res. 37, 127-153.

[108] Napoli, C., Lemieux, C. and Jorgensen, R. (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. Plant Cell 2, 279-289.

[109] Guo, S. and Kemphues, K.J. (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 81, 611-620.

[110] Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806-811.

[111] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494-498.

[112] Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. genes Dev 15, 188-200.

[113] Zamore, P.D. and Haley, B. (2005). Ribo-gnome: The Big World of Small RNAs. Science 309, 1519-1524.
[114] Nykanen, A., Haley, B. and Zamore, P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 107, 309-21.
[115] Tomari, Y., Du, T. and Zamore, P.D. (2007). Sorting of Drosophila Small Silencing RNAs. Cell 130, 299-308.
[116] Liu, J. et al. (2004). Argonaute2 Is the Catalytic Engine of Mammalian RNAi. Science 305, 1437-1441.
[117] Rivas, F.V., Tolia, N.H., Song, J.-J., Aragon, J.P., Liu, J., Hannon, G.J. and Joshua-Tor, L. (2005). Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Biol 12, 340-349.
[118] MacRae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D. and Doudna, J.A. (2006). Structural Basis for Double-Stranded RNA Processing by Dicer. Science 311, 195-198.
[119] Wei, Q., Lipardi, C. and Paterson, B.M. (2003). Analysis of the 3'-hydroxyl group in Drosophila siRNA function. Methods 30, 337-347.
[120] Lingel, A., Simon, B., Izaurralde, E. and Sattler, M. (2004). Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. Nat Struct Mol Biol 11, 576-7.
[121] Siolas, D., Lerner, C., Burchard, J., Ge, W., Linsley, P.S., Paddison, P.J., Hannon, G.J. and Cleary, M.A. (2005). Synthetic shRNAs as potent RNAi triggers. Nat Biotechnol 23, 227-31.
[122] Pei, Y. and Tuschl, T. (2006). On the art of identifying effective and specific siRNAs. Nat Meth 3, 670-676.
[123] Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R. and Hannon, G.J. (2001). Argonaute2, a Link Between Genetic and Biochemical Analyses of RNAi. Science 293, 1146-1150.
[124] Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. and Tuschl, T. (2002). Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi. Cell 110, 563-574.
[125] Tolia, N.H. and Joshua-Tor, L. (2007). Slicer and the Argonautes. Nat Chem Biol 3, 36-43.
[126] Martinez, J. and Tuschl, T. (2004). RISC is a 5' phosphomonoester-producing RNA endonuclease. Genes Dev 18, 975-980.
[127] Schwarz, D.S., Tomari, Y. and Zamore, P.D. (2004). The RNA-Induced Silencing Complex Is a Mg2+-Dependent Endonuclease. Current Biology 14, 787-791.
[128] Czauderna, F., Fechtner, M., Dames, S., Aygun, H., Klippel, A., Pronk, G.J., Giese, K. and Kaufmann, J. (2003). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. Nucleic Acids Res 31, 2705-16.
[129] Hutvagner, G. and Zamore, P.D. (2002). A microRNA in a Multiple-Turnover RNAi Enzyme Complex. Science 297, 2056-2060.
[130] Chiu, Y.L. and Rana, T.M. (2003). siRNA function in RNAi: a chemical modification analysis. Rna 9, 1034-48.
[131] Chiu, Y.-L. and Rana, T.M. (2002). RNAi in Human Cells: Basic Structural and Functional Features of Small Interfering RNA. Molecular Cell 10, 549-561.

[132] Eulalio, A., Behm-Ansmant, I., Schweizer, D. and Izaurralde, E. (2007). P-Body Formation Is a Consequence, Not the Cause, of RNA-Mediated Gene Silencing. Mol Cell Biol 27, 3970-3981.

[133] Khvorova, A., Reynolds, A. and Jayasena, S.D. (2003). Functional siRNAs and miRNAs Exhibit Strand Bias. Cell 115, 209-216.

[134] Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P.D. (2003). Asymmetry in the Assembly of the RNAi Enzyme Complex. Cell 115, 199-208.

[135] Cogoni, C. and Macino, G. (1999). Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature 399, 166-169.

[136] Lipardi, C., Wei, Q. and Paterson, B.M. (2001). RNAi as Random Degradative PCR: siRNA Primers Convert mRNA into dsRNAs that Are Degraded to Generate New siRNAs. Cell 107, 297-307.

[137] Schwarz, D.S., Hutvagner, G., Haley, B. and Zamore, P.D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol Cell 10, 537-48.

[138] Hu, X., Hipolito, S., Lynn, R., Abraham, V., Ramos, S. and Wong-Staal, F. (2004). Relative gene-silencing efficiencies of small interfering RNAs targeting sense and antisense transcripts from the same genetic locus. Nucleic Acids Res 32, 4609-4617.

[139] Amarzguioui, M., Holen, T., Babaie, E. and Prydz, H. (2003). Tolerance for mutations and chemical modifications in a siRNA. Nucleic Acids Res 31, 589-595.

[140] Du, Q., Thonberg, H., Wang, J., Wahlestedt, C. and Liang, Z. (2005). A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. Nucleic Acids Res 33, 1671-1677.

[141] Elmen, J. et al. (2005). Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. Nucleic Acids Res 33, 439-47.

[142] Mook, O.R., Baas, F., de Wissel, M.B. and Fluiter, K. (2007). Evaluation of locked nucleic acid-modified small interfering RNA in vitro and in vivo. Mol Cancer Ther 6, 833-843.

[143] Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. and Saigo, K. (2003). Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr Biol 13, 41-6.

[144] Kittler, R. et al. (2007). Genome-wide resources of endoribonuclease-prepared short interfering RNAs for specific loss-of-function studies. Nat Methods 4, 337-44.

[145] De Paula, D., Bentley, M.V. and Mahato, R.I. (2007). Hydrophobization and bioconjugation for enhanced siRNA delivery and targeting. Rna 13, 431-56.

[146] Whitehurst, A.W. et al. (2007). Synthetic lethal screen identification of chemosensitizer loci in cancer cells. Nature 446, 815-819.

[147] Ziauddin, J. and Sabatini, D.M. (2001). Microarrays of cells expressing defined cDNAs. Nature 411, 107-110.

[148] Ovcharenko, D., Jarvis, R., Hunicke-Smith, S., Kelnar, K. and Brown, D. (2005). High-throughput RNAi screening in vitro: From cell lines to primary cells. Rna 11, 985-993.

[149] Song, E. et al. (2003). RNA interference targeting Fas protects mice from fulminant hepatitis. Nat Med 9, 347-51.
[150] Braasch, D.A., Paroo, Z., Constantinescu, A., Ren, G., Oz, O.K., Mason, R.P. and Corey, D.R. (2004). Biodistribution of phosphodiester and phosphorothioate siRNA. Bioorganic & Medicinal Chemistry Letters 14, 1139-1143.
[151] Soutschek, J. et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432, 173-8.
[152] Layzer, J.M., McCaffrey, A.P., Tanner, A.K., Huang, Z.A.N., Kay, M.A. and Sullenger, B.A. (2004). In vivo activity of nuclease-resistant siRNAs. Rna 10, 766-771.
[153] Soutschek, J. et al. (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. Nature 432, 173-178.
[154] Heyes, J., Palmer, L., Bremner, K. and MacLachlan, I. (2005). Cationic lipid saturation influences intracellular delivery of encapsulated nucleic acids. Journal of Controlled Release 107, 276-287.
[155] Wheeler, J.J. et al. (1999). Stabilized plasmid-lipid particles: construction and characterization. Gene Ther 6, 271-81.
[156] Morrissey, D.V. et al. (2005). Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. Nat Biotechnol 23, 1002-7.
[157] Zimmermann, T.S. et al. (2006). RNAi-mediated gene silencing in non-human primates. Nature 441, 111-4.
[158] Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N. and Chen, J. (2004). Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc Natl Acad Sci U S A 101, 8676-8681.
[159] Schiffelers, R.M. et al. (2004). Cancer siRNA therapy by tumor selective delivery with ligand-targeted sterically stabilized nanoparticle. Nucleic Acids Res 32, e149-.
[160] Urban-Klein, B., Werth, S., Abuharbeid, S., Czubayko, F. and Aigner, A. (2004). RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. Gene Ther 12, 461-466.
[161] Chu, T.C., Twu, K.Y., Ellington, A.D. and Levy, M. (2006). Aptamer mediated siRNA delivery. Nucleic Acids Res 34, e73-.
[162] Hu-Lieskovan, S., Heidel, J.D., Bartlett, D.W., Davis, M.E. and Triche, T.J. (2005). Sequence-Specific Knockdown of EWS-FLI1 by Targeted, Nonviral Delivery of Small Interfering RNA Inhibits Tumor Growth in a Murine Model of Metastatic Ewing's Sarcoma. Cancer Res 65, 8984-8992.
[163] Song, E. et al. (2005). Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. Nat Biotechnol 23, 709-17.
[164] Kumar, P. et al. (2007). Transvascular delivery of small interfering RNA to the central nervous system. Nature 448, 39-43.
[165] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science 296, 550-3.
[166] Paddison, P.J., Caudy, A.A. and Hannon, G.J. (2002). Stable suppression of gene expression by RNAi in mammalian cells. Proc Natl Acad Sci U S A 99, 1443-8.

[167] Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16, 948-58.

[168] Czauderna, F. et al. (2003). Inducible shRNA expression for application in a prostate cancer mouse model. Nucleic Acids Res 31, e127.

[169] Kawasaki, H. and Taira, K. (2003). Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res 31, 700-7.

[170] Paul, C.P., Good, P.D., Li, S.X., Kleihauer, A., Rossi, J.J. and Engelke, D.R. (2003). Localized expression of small RNA inhibitors in human cells. Mol Ther 7, 237-47.

[171] Miyagishi, M., Sumimoto, H., Miyoshi H., Kawakami, Y. and Taira, K. (2004). Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. J Gen Med 6, 715-723.

[172] Paddison, P.J., Cleary, M., Silva, J.M., Chang, K., Sheth, N., Sachidanandam, R. and Hannon, G.J. (2004). Cloning of short hairpin RNAs for gene knockdown in mammalian cells. Nat Meth 1, 163-167.

[173] Hernandez-Hoyos, G., Alberola-Ila, J. and David R. Engelke, a.J.J.R. (2005). Analysis of T-Cell Development by Using Short Interfering RNA to Knock Down Protein Expression. Methods in Enzymology Volume 392, 199-217.

[174] Unwalla, H.J., Li, M.-J., Kim, J.D., Li, H.T., Ehsani, A., Alluin, J. and Rossi, J.J. (2004). Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. Nat Biotech 22, 1573-1578.

[175] Zeng, Y., Cai, X., Cullen, B.R. and David R. Engelke, a.J.J.R. (2005). Use of RNA Polymerase II to Transcribe Artificial MicroRNAs. Methods in Enzymology Volume 392, 371-380.

[176] Zhou, H., Xia, X.G. and Xu, Z. (2005). An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. Nucleic Acids Res 33, e62.

[177] Xia, H., Mao, Q., Paulson, H.L. and Davidson, B.L. (2002). siRNA-mediated gene silencing in vitro and in vivo. Nat Biotech 20, 1006-1010.

[178] Heinonen, J.E., Mohamed, A.J., Nore, B.F. and Smith, C.I. (2005). Inducible H1 promoter-driven lentiviral siRNA expression by Stuffer reporter deletion. Oligonucleotides 15, 139-44.

[179] Yang, S. and Hecht, N.B. (2004). Translin associated protein X is essential for cellular proliferation. FEBS Lett 576, 221-5.

[180] Xia, X.-G., Zhou, H., Samper, E., Melov, S. and Xu, Z. (2006) Pol II-Expressed shRNA Knocks Down Sod2 Gene Expression and Causes Phenotypes of the Gene Knockout in Mice, Plant physiol, 142, 429-440

[181] Jackson, D.A., Pombo, A.N.A. and Iborra, F. (2000). The balance sheet for transcription: an analysis of nuclear RNA metabolism in mammalian cells. Faseb J 14, 242-254.

[182] Noonberg, S.B., Scott, G.K., Garovoy, M.R., Benz, C.C. and Hunt, C.A. (1994). In vivo generation of highly abundant sequence-specific oligonucleotides for antisense and triplex gene regulation. Nucleic Acids Res 22, 2830-2836.

[183] He, Y., Zeng, Q., Drenning, S.D., Melhem, M.F., Tweardy, D.J., Huang, L. and Grandis, J.R. (1998). Inhibition of human squamous cell carcinoma growth in vivo by epidermal growth factor receptor antisense RNA transcribed from the U6 promoter. J Natl Cancer Inst 90, 1080-1087.
[184] Good, P.D. et al. (1997). Expression of small, therapeutic RNAs in human cell nuclei. Gene Ther 4, 45-54.
[185] Paul, C.P., Good, P.D., Winer, I. and Engelke, D.R. (2002). Effective expression of small interfering RNA in human cells. Nat Biotechnol 20, 505-8.
[186] Xia, X.G., Zhou, H., Ding, H., Affar el, B., Shi, Y. and Xu, Z. (2003). An enhanced U6 promoter for synthesis of short hairpin RNA. Nucleic Acids Res 31, e100.
[187] Hassani, Z., Francois, J.C., Alfama, G., Dubois, G.M., Paris, M., Giovannangeli, C. and Demeneix, B.A. (2007). A hybrid CMV-H1 construct improves efficiency of PEI-delivered shRNA in the mouse brain. Nucleic Acids Res 35, e65.
[188] Fewell, G.D. and Schmitt, K. (2006). Vector-based RNAi approaches for stable, inducible and genome-wide screens. Drug Discovery Today 11, 975-982.
[189] Chang, K., Elledge, S.J. and Hannon, G.J. (2006). Lessons from Nature: microRNA-based shRNA libraries. Nat Meth 3, 707-714.
[190] Chen, Y., Stamatoyannopoulos, G. and Song, C.-Z. (2003). Down-Regulation of CXCR4 by Inducible Small Interfering RNA Inhibits Breast Cancer Cell Invasion in Vitro. Cancer Res 63, 4801-4804.
[191] Ohkawa, J. and Taira, K. (2000). Control of the Functional Activity of an Antisense RNA by a Tetracycline-Responsive Derivative of the Human U6 snRNA Promoter. Hum Gen Ther 11, 577-585.
[192] van de Wetering, M. et al. (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. EMBO Rep 4, 609-15.
[193] Szulc, J., Wiznerowicz, M., Sauvain, M.-O., Trono, D. and Aebischer, P. (2006). A versatile tool for conditional gene expression and knockdown. Nat Meth 3, 109-116.
[194] Hamdorf, M., Muckenfuss, H., Tschulena, U., Pleschka, S., Sanzenbacher, R., Cichutek, K. and Flory, E. (2006). An inducible T7 RNA polymerase-dependent plasmid system. Mol Biotechnol 33, 13-21.
[195] Gupta, S., Schoer, R.A., Egan, J.E., Hannon, G.J. and Mittal, V. (2004). From the Cover: Inducible, reversible, and stable RNA interference in mammalian cells. Proc Natl Acad Sci U S A 101, 1927-1932.
[196] Van Craenenbroeck, K., Vanhoenacker, P., Leysen, J.E. and Haegeman, G. (2001). Evaluation of the tetracycline- and ecdysone-inducible systems for expression of neurotransmitter receptors in mammalian cells. Eur Neuro Sci 14, 968-976.
[197] Hoess, R.H., Ziese, M. and Sternberg, N. (1982). P1 Site-Specific Recombination: Nucleotide Sequence of the Recombining Sites. Proc Natl Acad Sci U S A 79, 3398-3402.
[198] Abremski, K. and Hoess, R. (1985). Phage P1 Cre-loxP site-specific recombination : Effects of DNA supercoiling on catenation and knotting of recombinant products. Journal of Molecular Biology 184, 211-220.

[199] Tiscornia, G., Tergaonkar, V., Galimi, F. and Verma, I.M. (2004). CRE recombinase-inducible RNA interference mediated by lentiviral vectors. Proc. Natl. Acad. Sci. 101, 7347-51.

[200] Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Van Parijs, L., Jaenisch, R. and Jacks, T. (2004). Cre-lox-regulated conditional RNA interference from transgenes. Proc. Natl. Acad. Sci. 101, 10380-5.

[201] Fritsch, L., Martinez, L.A., Sekhri, R., Naguibneva, I., Gerard, M., Vandromme, M., Schaeffer, L. and Harel-Bellan, A. (2004). Conditional gene knock-down by CRE-dependent short interfering RNAs. EMBO Rep. 5, 178-82.

[202] Kasim, V., Miyagishi, M. and Taira, K. (2004). Control of siRNA expression using the Cre-loxP recombination system. Nucleic Acids Res. 32, e66.

[203] Wadia, J.S., Stan, R.V. and Dowdy, S.F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med 10, 310-315.

[204] Jacob, J. and Baltimore, D. (1999). Modelling T-cell memory by genetic marking of memory T cells in vivo. Nature 399, 593-597.

[205] Feil, R., Brocard, J., Mascrez, B., LeMeur, M., Metzger, D. and Chambon, P. (1996). Ligand-activated site-specific recombination in mice. proc Natl Acad Sci U S A 93, 10887-10890.

[206] Murphy, G.J., Mostoslavsky, G., Kotton, D.N. and Mulligan, R.C. (2006). Exogenous control of mammalian gene expression via modulation of translational termination. Nat Med 12, 1093-1099.

[207] Stevenson, M. (2004). Therapeutic potential of RNA interference. N Engl J Med 351, 1772-7.

[208] Connelly, J.C., Kirkham, L.A. and Leach, D.R.F. (1998). The SbcCD nuclease of Escherichia coli is a structural maintenance of chromosomes (SMC) family protein that cleaves hairpin DNA. Proc Natl Acad Sci U S A 95, 7969-7974.

[209] Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843-854.

[210] Bartel, D.P. (2004). MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. Cell 116, 281-297.

[211] Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L. and Iggo, R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet 34, 263-4.

[212] Samuel, C.E. (2001). Antiviral Actions of Interferons. Clin Microb Rev 14, 778-809.

[213] Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H. and Williams, B.R. (2003). Activation of the interferon system by short-interfering RNAs. Nat Cell Biol 5, 834-9.

[214] Heidel, J.D., Hu, S., Liu, X.F., Triche, T.J. and Davis, M.E. (2004). Lack of interferon response in animals to naked siRNAs. Nat Biotechnol 22, 1579-82.

[215] Boutla, A., Delidakis, C., Livadaras, I., Tsagris, M. and Tabler, M. (2001). Short 5'-phosphorylated double-stranded RNAs induce RNA interference in Drosophila. Curr Biol 11, 1776-80.

[216] Birmingham, A. et al. (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Methods 3, 199-204.

[217] Jackson, A.L. et al. (2003). Expression profiling reveals off-target gene regulation by RNAi. Nat Biotechnol 21, 635-7.
[218] Lin, X., Ruan, X., Anderson, M.G., McDowell, J.A., Kroeger, P.E., Fesik, S.W. and Shen, Y. (2005). siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. Nucleic Acids Res 33, 4527-4535.
[219] Buchholz, F., Kittler, R., Slabicki, M. and Theis, M. (2006). Enzymatically prepared RNAi libraries. Nat Methods 3, 696-700.
[220] Jackson, A.L., Burchard, J., Schelter, J., Chau, B.N., Cleary, M., Lim, L. and Linsley, P.S. (2006). Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. Rna 12, 1179-87.
[221] Dorsett, Y. and Tuschl, T. (2004). siRNAs: applications in functional genomics and potential as therapeutics. Nat Rev Drug Discov 3, 318-29.
[222] Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 303, 1529-31.
[223] Heil, F. et al. (2004). Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 303, 1526-9.
[224] Judge, A.D., Sood, V., Shaw, J.R., Fang, D., McClintock, K. and MacLachlan, I. (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. Nat Biotechnol 23, 457-62.
[225] Judge, A.D., Bola, G., Lee, A.C. and MacLachlan, I. (2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. Mol Ther 13, 494-505.
[226] Morrissey, D.V. et al. (2005). Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. Hepatology 41, 1349-56.
[227] Jackson, A.L. et al. (2006). Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing. Rna 12, 1197-205.
[228] Xu, P., Zhang, Y., Kang, L., Roossinck, M.J. and Mysore, K.S. (2006). Computational Estimation and Experimental Verification of Off-Target Silencing during Posttranscriptional Gene Silencing in Plants. Plant Physiol 142, 429-440.
[229] Boden, D., Pusch, O., Lee, F., Tucker, L. and Ramratnam, B. (2003). Human Immunodeficiency Virus Type 1 Escape from RNA Interference. J Virol 77, 11531-11535.
[230] Gitlin, L., Stone, J.K. and Andino, R. (2005). Poliovirus Escape from RNA Interference: Short Interfering RNA-Target Recognition and Implications for Therapeutic Approaches. J Virol 79, 1027-1035.
[231] Wilson, J.A. and Richardson, C.D. (2005). Hepatitis C Virus Replicons Escape RNA Interference Induced by a Short Interfering RNA Directed against the NS5b Coding Region. J Virol 79, 7050-7058.
[232] Grimm, D. and Kay, M.A. (2007). Combinatorial RNAi: a winning strategy for the race against evolving targets? Mol Ther 15, 878-88.
[233] Chung, K.-H., Hart, C.C., Al-Bassam, S., Avery, A., Taylor, J., Patel, P.D., Vojtek, A.B. and Turner, D.L. (2006). Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. Nucleic Acids Res 34, e53-.

[234] Song, E. et al. (2003). Sustained Small Interfering RNA-Mediated Human Immunodeficiency Virus Type 1 Inhibition in Primary Macrophages. J Virol 77, 7174-7181.

[235] Jarczak, D., Korf, M., Beger, C., Manns, M.P. and Kruger, M. (2005). Hairpin ribozymes in combination with siRNAs against highly conserved hepatitis C virus sequence inhibit RNA replication and protein translation from hepatitis C virus subgenomic replicons. Febs J 272, 5910-5922.

[236] Unwalla, H.J., Li, H.-T., Bahner, I., Li, M.-J., Kohn, D. and Rossi, J.J. (2006). Novel Pol II Fusion Promoter Directs Human Immunodeficiency Virus Type 1-Inducible Coexpression of a Short Hairpin RNA and Protein. J Virol 80, 1863-1873.

[237] Hornung, V. et al. (2005). Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. Nat Med 11, 263-70.

[238] Kawakami, Y., Kitaura, J., Hata, D., Yao, L. and Kawakami, T. (1999). Functions of Bruton's tyrosine kinase in mast and B cells. J Leuk Biol 65, 286-290.

[239] Nore, B.F. et al. (2000). Redistribution of Bruton's tyrosine kinase by activation of phosphatidylinositol 3-kinase and Rho-family GTPases. Eur J Immunol 30, 145-154.

[240] Turner, H. and Kinet, J.P. (1999). Signalling through the high-affinity IgE receptor Fc epsilonRI. Nature 402, B24-30.

[241] Miller, A.T. and Berg, L.J. (2002). New insights into the regulation and functions of Tec family tyrosine kinases in the immune system. Current Opinion in Immunology 14, 331-340.

[242] Ji, J., Wernli, M., Klimkait, T. and Erb, P. (2003). Enhanced gene silencing by the application of multiple specific small interfering RNAs. FEBS Letters 552, 247-252.

[243] Boden, D., Pusch, O., Lee, F., Tucker, L., Shank, P.R. and Ramratnam, B. (2003). Promoter choice affects the potency of HIV-1 specific RNA interference. Nucleic Acids Res. 31, 5033-8.

[244] Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W. and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. Science 268, 1766-9.

[245] Hasuwa, H., Kaseda, K., Einarsdottir, T. and Okabe, M. (2002). Small interfering RNA and gene silencing in transgenic mice and rats. FEBS Lett. 532, 227-30.

[246] Kawakami, Y. et al. (2006). Regulation of dendritic cell maturation and function by Bruton's tyrosine kinase via IL-10 and Stat3. Proc Natl Acad Sci U S A 103, 153-8.

[247] Gubina, E., Luo, X., Kwon, E., Sakamoto, K., Shi, Y.F. and Mufson, R.A. (2001). betac cytokine receptor-induced stimulation of cAMP response element binding protein phosphorylation requires protein kinase C in myeloid cells: a novel cytokine signal transduction cascade. J Immunol 167, 4303-10.

[248] Shankar, D.B. et al. (2005). The role of CREB as a proto-oncogene in hematopoiesis and in acute myeloid leukemia. Cancer Cell 7, 351-62.

[249] Bence, K., Ma, W., Kozasa, T. and Huang, X.-Y. (1997). Direct stimulation of Bruton's tyrosine kinase by Gq-protein [alpha]-subunit. 389, 296-299.

[250] Tsukada, S., Simon, M.I., Witte, O.N. and Katz, A. (1994). Binding of {beta}{gamma} Subunits of Heterotrimeric G Proteins to the PH Domain of Bruton Tyrosine Kinase. Proc Natl Acad Sci U S A 91, 11256-11260.
[251] Dizier, M.H., Bouzigon, E., Guilloud-Bataille, M., Genin, E., Oryszczyn, M.P., Annesi-Maesano, I. and Demenais, F. (2007). Evidence for a locus in 1p31 region specifically linked to the co-morbidity of asthma and allergic rhinitis in the EGEA study. Hum Hered 63, 162-7.
[252] Laitinen, T. et al. (2004). Characterization of a Common Susceptibility Locus for Asthma-Related Traits. Science 304, 300-304.