Elucidating the Role of Effector Caspases in Immune Development Using Lentiviral RNAi

By

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B.S. Biochemistry
University of California, Davis, 2000

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ABSTRACT

Caspases play an important role in apoptosis, or programmed cell death. In particular, three highly related effector caspases, caspases-3, -6, and –7, translate upstream death signals into the physical manifestations of apoptosis by proteolytically cleaving structural and enzymatic targets. However, it is not clear what specific role each individual caspase plays in apoptosis or whether interaction between them is important. We used RNA interference (RNAi) to examine their roles in immune cells.

RNAi has revolutionized the field of mammalian genetics by expediting the interrogation of gene function. Endogenous genes are targeted for silencing by the introduction of double stranded RNAs, known as short interfering RNAs (siRNAs), through a mechanism that is well conserved across many species. While this technique has been used successfully in tissue culture experiments, our studies focused on extending the use of RNAi into immune cells. Our initial experiments demonstrated that primary T cells were capable of RNAi-based gene silencing, but were difficult to introduce siRNAs into. Therefore, more robust techniques for the stable and efficient introduction of siRNAs into primary immune cells and animal models were required. Viral vectors, which can infect a wide variety of cell types and drive consistent transgene expression, provide a potential delivery vehicle for short hairpin RNAs (shRNAs), an alternative form of double stranded RNA produced within the target cell. Thus, we designed a lentiviral vector system for delivering shRNAs and used the vector to generate transgenic knockdown animals. Further experiments enhanced this vector system by enabling tissue- or temporal-specific transgene or shRNA expression as well as reducing variegated viral expression.

Using these lentiviral RNAi vectors, we began to assess the role of effector caspases in the immune system. We generated T cell lines in which the effector caspases were ablated individually or simultaneously by RNAi and tested whether these cells were resistant to apoptosis. Of the three effector caspases, only silencing of caspase-3 protected against cell death in T cells, whereas simultaneous knockdown of caspase-6 or caspase-7 with caspase-3 provided no additional protective effect against apoptosis. We also generated transgenic caspase-7 knockdown animals and found that this caspase might influence B cell development.

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Chapter 1

Introduction and Background

Introduction

Caspases play an important role in apoptosis, or programmed cell death. In particular, three highly related effector caspases, caspases-3, -6, and –7, translate upstream death signals into the physical manifestations of apoptosis by proteolytically cleaving structural and enzymatic targets. However, it is not clear what specific role each individual caspase plays in apoptosis or whether interaction between them is important. As more gene families are discovered, the ability to assign roles to each individual family member is becoming more difficult. Analysis of a single gene knockout from a large gene family can be obfuscated by the potential redundancy of non-targeted family members. Breeding for multiple gene knockouts is time consuming, complex, and can provide limited insight into gene function as compound mutants frequently show early lethality. A promising new technique, which co-opts an endogenous cellular process known as RNA interference (RNAi), could revolutionize multigene analysis. This method introduces into cells small double-stranded RNAs (dsRNAs) homologous to targeted sequences in order to eliminate gene products. The ultimate success of this technique will depend on designing effective RNAi sequences and efficiently delivering these short interfering RNAs (siRNAs) into mammalian cells of interest. If successful, RNAi could prove a promising alternative to traditional genetic manipulations for the creation of complex *in vivo* genetic models.

Background

RNA interference – a new paradigm for genetic regulation

RNAi, originally described in the worm *C. elegans* by Fire and colleagues, is a well conserved endogenous pathway for regulating gene expression in many organisms[1, 2]. This process, which uses a class of short non-coding RNA known as microRNAs, coordinates many vital cellular functions in addition to translational regulation. Since scientists are co-opting this natural pathway to silence genes of interest, the design of effective and specific silencing sequences depends on a thorough understanding of the endogenous RNAi mechanism.

While many of the specific details are still being elucidated, the basic mechanism of RNA interference is grossly understood. In the nucleus of the cell, RNA polymerase II promoters transcribe single stranded RNA precursors up to 2kb long which adopt secondary structures to create double stranded stem loops [3, 4]. These precursors, known as pri-micro-RNAs, are cleaved by the ribonuclease Drosha into an exportable pre-micro-RNAs[5]. After export to the cytoplasm, the pre-micro-RNA is cleaved by the enzyme Dicer into a 20-23 base pair (bp) double-stranded RNA known as micro-RNA(miRNA) [6], which is subsequently incorporated into the RNA induced silencing complex, or RISC [7]. While unwinding of double-stranded RNA and the stable incorporation of one strand of a short RNA duplex into the RISC is mediated by a Dicer/R2D2 complex in flies, no R2D2 homologue has yet been found in mammalian cells [8, 9]. While other components of the mammalian RISC are still being identified, Argonaute is the only component found in all RISC complexes and is responsible for the silencing of the target mRNA [10-16]. Since they are generally have imperfect

homology to their target, miRNAs usually create bulges when they anneal to target mRNA in the RISC [7, 17, 18]. A target mRNA will have several different miRNA binding sites in its 3' UTR, suggesting that several miRNAs might coordinately control mRNA translation [17, 19]. While the detailed mechanism is unclear, it appears that gene silencing results from the binding of RISC to an existing mRNA, thereby preventing its translation. It remains unclear if the block occurs before translational initiation or at a later point before the complete translation of full-length protein. It remains possible that both models of silencing could be correct and that there may be a miRNA or species specificity which determines when this blocks occurs [20-22].

When RNAi is used as a research tool, a different mechanism of gene silencing is invoked. Introduction of dsRNAs that are perfectly homologous to the target mRNA leads to cleavage and degradation of the target mRNA by the RISC complex, thereby preventing translation of the encoded protein [23, 24]. In contrast to the endogenous method of translational suppression, effective silencing through this method usually requires only a single siRNA binding site in the target mRNA[17]. It is still unclear, however, whether both methods utilize the same RISC complex, or if different components are used for each type of silencing [25].

With the exception of *Saccharomyces cerevisiae,* most model organisms have the enzymes required for RNAi [26]. Despite the widespread conservation of this regulatory mechanism, specific pathway variations exist between organisms. For instance, *C. elegans* possess a RNA-dependent RNA polymerase, enabling dsRNA amplification and long term silencing[27, 28]. While long double stranded RNA can direct silencing in both worms and flies [29], the introduction of dsRNA longer than 30bp into mammalian

cells leads to cell death through the interferon response, which hindered the early application of RNAi in mammalian model systems.

The first successful application of RNAi in mammalian cells occurred in 2001 when Tuschl and colleagues showed that dsRNA shorter than 30 bp could induce silencing in mammalian cells [30]. Their work focused on 21 bp dsRNAs termed short interfering RNAs (siRNAs), with 19 bp of homology and 2 nucleotide (nt) 3' overhangs. However, there were still challenges to using siRNAs in mammalian cells. Identifying effective sequences for silencing was a slow trial and error process since only a fraction of the sequences derived from a particular mRNA worked as effective targets [31]. Information from structural analysis of the RNAi proteins Dicer and Argonaute along with systematic analysis of all targeting sequences within genes enabled the creation of rational design algorithms that could more efficiently identify effective sequences for silencing [8, 32-34]. Testing of these rational design algorithms found that over 50% of the sequences identified using the algorithms were highly effective compared to only 17% of randomly selected sequences [33]. By taking into account complex interactions between parameters, a second generation of rational design algorithms using artificial neural networks have further improved the efficiency of sequence selection [35].

In addition to sequence design difficulties, temporal limitations restrict the effective use of siRNAs in many mammalian cell types. Since mammalian cells lack the RNA-dependent RNA polymerase found in more primitive organisms, silencing efficiency in mammalian cells is reduced through degradation and dilution of siRNAs during cell proliferation [27, 28, 36]. To circumvent this issue, several groups designed vectors capable of stably producing short dsRNAs within cells of interest [37-39]. These

vectors used a RNA polymerase III promoter to drive expression of short hairpin RNAs (shRNAs), doubled-stranded RNA whose sense and anti-sense strands are separated by a stem loop forming a hairpin structure [40]. This hairpin, which mimics the natural structure of pre-miRNA, targets the shRNA to Dicer for processing into siRNA [37]. Since the exact structural requirements for Dicer substrates were unclear, the pol III promoter was selected to drive shRNA expression[37]. Unlike polymerase II transcription where poly (A) polymerase adds a poly (A) tail of indeterminate length, RNA polymerase III has defined start and stop signals that result in transcripts that form identical and well-characterized dsRNA structures [41]. DNA based vectors using either the pol III U6 or H1 promoters proved efficient *in vitro*, and the inclusion of selectable markers made this a viable approach for long-term silencing in tissue culture [42, 43].

While DNA based vectors work well in most cell types, primary cells are notoriously difficult to transfect. Cationic liposomal agents, whose efficiencies are high in cultured lines, have low transfection rates in primary cells [44] and physical methods for DNA delivery, such as electroporation, markedly reduce the viability of treated cells [36, 45, 46]. To introduce exogenous DNA into primary cells, researchers adapted the pol III shRNA technology for delivery by retroviral vectors [47], already widely used for gene delivery applications [48]. Integration of the viral genome into the target cell ensures that expression of the shRNA will be stable over long periods of time and the retroviral machinery enables cells to be infected with high efficiency [48]. The lentiviruses, a particular subgroup of retroviruses, can be used to infect embryos and generate animals with high levels of transgene expression [49]. Our research group, as

well as others, constructed a lentiviral vector capable of generating shRNAs within cells in order to validate this method for producing transgenic RNAi animals [50-53].

Lentiviruses for gene delivery

Retroviruses are a family of postive-stranded enveloped RNA viruses that replicate through a DNA intermediate which integrates into the genome of a host cell [54]. The retroviral virion consists of a 7-12 kb RNA encapsulated by a protein core known as the capsid. As the virus matures, the capsid is enveloped by a lipid bilayer membrane obtained from the host cell [54]. The surface glycoproteins which populate the viral envelope determine the tropism of the virus and enable infection by interacting with elements of the host cell's membrane [55]. Once inside the target cell, the viral genome is converted to double stranded DNA in a process known as reverse transcription [56, 57]. The viral protein integrase directs the insertion of the viral DNA into the host cell's genome where it serves as a stable and long-term template for expressing additional copies of the genome and the viral proteins required for producing additional virions [58]. Assembled virions are shed from the host's cell membrane through a process known as budding [54].

All retroviral genomes contain four genes which encode the essential proteins for viral replication; gag, pol, pro, and env [54]. Each gene encodes a polypeptide chain that is proteolytically cleaved into the individual proteins required for the viral lifecyle [59]. Gag encodes for the capsid (CA), nucelocapsid (NC), and matrix (MA) proteins that form the isohedral capsid in which the viral RNA is packaged [60, 61]. Two proteins, reverse transcriptase and integrase, are derived from pol. Reverse transciptase generates a DNA

copy of the viral RNA and integrase inserts the provirus into the host cell's genome [62]. Transcription from pro leads to the expression of a protease thought to be important in the late stages of viral packaging [58]. Env is cleaved into two proteins, SU and TM, which form the glycoprotein required for recognizing cell surface receptors and entering host cells [63]. Variable regions in in the SU protein largely determine the host specificity of a particle retrovirus [54].

Retroviruses are categorized broadly as simple or complex. Simple retroviruses contain only gag, pol, pro, and env genes, while complex retroviruses, such as lentiviruses, contain additional regulatory sequences [64]. For example, Rev, an HIV protein, alters splicing and enables the full-length viral RNA to be expressed [65, 66]. The accessory genes of lentiviruses enable them to infect non-cycling and quiescent cells, such as neurons and stem cells [67]. While simple retroviruses require the nuclear membrane to break down in order for the viral DNA to access the host DNA, lentiviruses use the central polypurine tract (cPPT) to target the viral DNA for importation through the nuclear membrane [68]. Despite differences in their regulation, both simple and complex retroviruses use the same core proteins and therefore function in fundamentally similar ways.

Since expression from integrated proviruses is stable, retroviruses can make ideal gene transfer vehicles [69]. Retroviral transfer vectors can be generated by removing the packaging genes (gag,pol,pro, and env) from the retroviral genome and placing them into separate vectors, leaving space for the insertion of transgenes for delivery into target cells [70]. Efficient and well-characterized murine retroviruses such as Moloney leukemia virus (MLV) and murine stem cell virus (MSCV) have been used as transfer vectors for

many years, especially in the study of immune cells [48]. Early attempts to generate transgenics animals using these viruses were only partially successful. Pre-implantation embryos were permissive for infection by MLV and viral integrants were capable of being transmitted in the germline through breeding as evidenced by Southern Blot [71]. However, transgene expression from these viral integrants was suppressed [72, 73]. Long term silencing is believed to be mediated by DNA methylation, whereas short term silencing is effected through transcriptional repression of elements in the viral LTR [73]. These mechanisms are thought to be evolved as defenses to restrict the expansion and spread of endogenous retroviruses [74]. Since lentiviruses are not known to exist endogenously in any mammalian genome, they are likely to be less susceptible to silencing [49].

Most of the lentiviruses used in research are derivatives of human immunodeficiency virus (HIV) [75], the prototypical member of the lentivirus family. Other vector systems based on the Equine infectious anemia virus (EIAV) and the Feline immunodeficiency virus (FIV) are becoming more widely used [76] since there are safety concerns using HIV-based vector systems. Manipulations, such as deleting or replacing regulatory elements with genes of non-lentiviral origin, have helped make HIV-based vectors safer and more efficient [77]. For instance, the U3 region of the 5' LTR was replaced by the strong promoter CMV to improve viral titer [78, 79]. Also, a deletion in the 3' LTR disables HIV LTR promoter activity after reverse transcription resulting in self-inactivation [80]. Disabling the endogenous HIV promoter also enables insertion of a different promoter between the LTRs to express a protein of interest [77]. Together,

these changes create a replication incompetent virus, which can only produce virus when the packaging proteins are provided in *trans* [76].

After the development of improved HIV vector system, several groups attempted to generate transgenic animals by infecting embryos with lentivirus. Since lentiviruses are not silenced during embryonic development, they generate transgenic mice which stably express a gene of interest[49, 71]. However, animals with several proviral integrants showed more consistent expression than those with a single copy, suggesting that some silencing mechanisms might be active [49, 81]. Expression from the viral transgene seems to pass to offspring through breeding, suggesting that larger cohorts may readily be bred for disease incidence studies [82]. This technique can be used to generate transgenics from strains or species, such as the rat, that are impervious to traditional pronuclear injection techniques [83]. In addition, lentiviral vectors can be used to create transgenics directly in strains where significant backcrossing would otherwise be required to achieve the desired genotype, such as the non-obese diabetic (NOD) strain [82].

Since lentiviruses show promise as a new transgenic technique, our goal was to integrate this new gene delivery tool with RNAi technology to accelerate the generation of knockdown animals. In particular, we thought that lentiviral RNAi technology would prove useful for the study of apoptosis in immune cells.

Caspases – executioners of apoptosis

The development of a complex multicellular organism requires the elimination of unwanted cells without disturbing the ongoing development of surrounding cells. The quick and safe disposal of unwanted cells is accomplished through a well organized

process known as apoptosis[84]. Apoptosis is characterized by a number of morphological and biochemical changes including membrane blebbing, chromatin condensation, and exposure of phosphatidylserine on the cell surface which serves as a signal for cell removal through phagocytosis by scavenging cells[84-86]. The activation and execution of the apoptotic pathway relies on an important family of proteins known as caspases. Caspases are specialized proteases that integrate upstream signals and translate them into the physical manifestations of apoptosis by cleaving downstream targets. Since the function of the prototypical family member ced-3 was first recognized in *C. elegans*, specifying the roles of individual caspases in apoptosis has been the focus of considerable research [87].

Caspases are a conserved gene family found in almost all metazoan organisms. The family has diversified from a single caspase in *C. elegans* to seven in *Drosophila* caspases and 11 in humans [88]. The term caspase, short for cysteine aspartate protease, refers to the fact that these enzymes use a catalytically active cysteine to direct cleavage in target proteins after aspartate residues [89]. Caspases are expressed as inactive zymogens composed of multiple domains. In addition to a prodomain, all caspases have a large (17-20 kilodalton [kd]) and a small subunit (10-14 kd) separated by a short spacer whose excision results in caspase activation. The mature enzyme is a homodimer of two activated heterodimers composed of the large and small caspase subunits [90, 91].

Based on their physical structure, the nine members of the murine caspase family can be broadly categorized into initiator and effector caspases. Initiator caspases, such as caspases-2,-8,and-9, have long prodomains 100 to 220 amino acids in length. These long prodomains contain protein-protein binding motifs that recruit initiator caspases into

specialized activating structures. For example, caspase-9 binds to Apaf-1 and cytochrome C to create the "apoptosome [92, 93]." Caspase zymogens within these activating structures are brought into close proximity to induce activating interchain (transcatalytic) cleavage between them[94, 95]. Once activated, initiator caspases are released from the activating structure into the cytoplasm where they can bind, cleave, and activate their target effector caspases, such as caspases-3, -6, and –7 [94, 96-98]. In contrast to initiator caspases, the short prodomain of effector caspases do not appear to play a role in activation. After activation, effector caspases cleave numerous cellular targets to turn off pathways that promote cell survival and activate pathways which lead to cellular dismantling. Targets of effector caspases include ICAD (an inhibitor to the DNAse CAD), PARP (the catalytic subunit of the DNA dependent protein kinase), and structural proteins such as laminins [99].

Recently, research has focused on the non-apoptotic roles of caspases including cytokine maturation, immune receptor signaling, and regulation of the cell cycle. In mice, caspases-1,-11, and –12 are thought to be involved in the maturation of interleukin IL-1β, an important inflammatory cytokine[100]. However, it was unclear how the non-apoptotic functions of caspases were mechanistically separated from apoptotic ones. Apoptosis normally follows caspase activation, suggesting that caspases must be regulated post-activation to fulfill their non-apoptotic roles. A family of proteins known as the inhibitors of apoptosis (IAPs) is known to inhibit caspases by occluding the caspases's active site [101]. It was recently postulated that the activating structures also regulate caspase activity by holding initiator caspases in active conformation without cleavage and by restricting the subcellular location of active enzyme [102]. In this case,

the caspase could only cleave specific localized targets and would be inert upon release from the activating structure.

The role of caspases in development has been investigated using knockout mice. However, knockouts of certain caspases, such as caspase-9, have been relatively unhelpful in elucidating caspase function. Caspase-9 knockouts usually die *in utero* when the skull fails to enclose ectopic brain tissue [103, 104]. Knocking out caspase-3, which acts downstream of caspase-9, results in animals that die from neuronal hyperplasia, although the defect is usually less severe than in the caspase-9 knockouts [105]. Caspase-2 and –6 knockouts are both viable and reached adulthood with no gross phenotypic abnormalities [106-108]. Further characterization of caspase-2 knockout cells suggested that caspase-2 influences neuronal cell death [109]. Caspase-7 knockouts die too early to be characterized and caspase-8 knockouts die on day E10.5 of apparent cardiac muscle malformations [106, 110]. Conditional knockouts of caspase-8 suggest involvement of caspase-8 in lymphocyte development [111]. To date, caspase knockouts seem to have defects within particular tissues rather than systemic defects, however, it is unclear whether this reflects caspase specialization or variances of expression between tissues.

Since mammals have multiple caspases, it is possible that redundancy or compensation of function might exist between caspase family members to ensure essential apoptosis proceeds even if one caspase is disabled. For instance, caspase-3 knockouts on a C57/BL6 background survive well past birth, while the same knockout on a sv/129 background dies perinatally [112]. In the C57/BL6 animals, caspase-7 appears responsible for the elimination of ectopic brain tissue in the absence of caspase-3 [113].

When stimulated with Fas antibody, caspase-6 and –7 activation was observed in the livers of caspase-3 and –9 knockout mice, demonstrating compensatory caspase activation in the absence of normal caspase pathways [114]. Marsden et al. demonstrate that in the absence of caspase-9, caspase-7 might be activated when apoptosis through the mitochondria is induced [115]. To determine the extent of these alternative activation pathways, double or triple caspase-knockout animals need to be generated and examined for effects on apoptosis.

The specific role of the executioner caspases individually and synergistically in immune cells and *in vivo* remains unclear. This thesis examines RNAi as a tool to probe caspase function, both within a single *in vitro* system and in a defined genetic background *in vivo*. The ability to integrate RNAi inducing hairpins into viruses would enable the combinatorial targeting of multiple caspases, which could help better define their roles *in vivo*.

Chapter 2

siRNA and shRNA delivery into immune cells

Attribution

I performed all the experiments with siRNAs in primary T cells. Douglas Rubinson cloned the LentiLox series of vectors, including LentiLox 3.7. I cloned the CD8 shRNA into LentiLox 3.7 and performed all the *in vitro* and primary T cell analysis. Michael McManus did the small RNA northerns, and Claudia Sievers did the northern for CD8 and CD4 mRNA expression. Doug Rubinson and Adam Kwiatkowski infected and cultured the ES cells use to generate chimeras, and a group at Biogen led by Martin Scott generated and provided images of the transgenic animals. Doug and I dissected the LentiLox derived animals and I ran and analyzed the FACS from all of the animals.

Introduction

One of the challenges in immunological research is the relative resistance of immune cells to manipulation. Naïve lymphocytes are virtually impossible to transfect with nucleic acids, a method that has helped elucidate biochemical pathways in many other cell types. Germline manipulation to generate gene knockouts seemed the only possible route for investigating the immune system. Animal models from the immune system often use specific receptor transgenes to simplify the analysis of immune genes and functions. Breeding genetic knockouts onto these transgenic strains can be time consuming because of the need to minimize strain differences. Furthermore, knockouts ablate protein expression throughout development, making it difficult to determine whether a particular phenotype resulted from changes during development or in the function of the mature cell. Thus, it would be beneficial to manipulate mature immune cells, preferably naïve cells not previously activated before, to clearly illuminate the role

of an altered gene. As the use of RNAi became more prevalent in mammalian cell culture, we saw an opportunity to use this technique in immune cells. My collaborators, Michael McManus and Brian Haines, demonstrated that delivery of siRNAs into T cell lines using electroporation was possible, indicating that RNAi was an active process in immune cells. At the time, it was not known if RNAi was universal in all mammalian cell types. Thus, my work focused on extending their results using electroporation to *ex vivo* primary cells.

Once it was established that primary T cells were capable of RNAi, we sought a more efficient and less toxic way of delivering these nucleic acids to the cells. An important discovery by Brummelkamp and colleagues showed that siRNAs could be generated within cells by expressing short hairpin RNAs (shRNAs) from DNA plasmids [37]. We thought that shRNAs adapted for expression from viral vectors would make an ideal delivery system for RNAi into immune cells. Our laboratory had vast experience using retroviral vectors, but was starting to focus on lentiviral vectors since they had several advantages over other retroviral vectors. First, they do not require a cell to be in cycle for infection, which suggests they could infect naïve lymphocytes. Recent reports demonstrate that these vectors can be used to generate transgenic animals through direct infection of embryos or ES cells[49, 116]. In contrast, transgenic animals made using retroviruses experienced silencing of the retroviral transgenes within weeks of infection even when the DNA methylases were ablated [71, 73]. We thought integration of shRNAs into lentiviral vectors would allow us to more efficiently manipulating immune cells. Additionally, we sought to generate a single viral system capable of infecting multiple cell types to avoid revalidation of targeting dsRNAs in a different delivery

vehicle for new experiments. We created a bifunctional lentiviral vector for RNAi called pLentiLox 3.7, which we then validated in tissue culture, primary cells, and *in vivo* by transgenesis.

Methods

Mice

Several strains of mice were used for the experiments described in this chapter. Primary splenocytes were isolated from DO11.10 TCR transgenics (a generous gift from Dr. C. London, University of California, Davis, CA), which expressed a transgenic TCR that recognizes ovalbumin (OVA) peptide presented on MHC class II molecules, and used for siRNA experiments. Lentiviral infections for validation of the LentiLox vector were performed on primary splenocytes from OT-I Rag1-/- animals, generously provided by Jianzhu Chen (Massachusetts Institute of Technology). ES cells from the AK7 line were used to generate lentiviral chimeric animals by injecting infected cells into 129 $Rag2^{-/-}$ blastocysts. Lentiviral transgenics were generated with C57/BL6 mice (Taconic). Mice were maintained in specific pathogen free facilities. All the experiments were carried out according to the guidelines established by the Committee on Animal Care at the Massachusetts Institute of Technology or the Institutional Animal Care and Use Committee at Biogen.

Cell culture

E10 is an immature double-positive thymocyte line derived from a TCR- and p53 double-mutant mouse on a mixed 129/Sv x C57BL/6 background [117]. These cells were

maintained at a maximal concentration of 2 x 10^6 cells/ml and were propagated in complete medium: DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. Primary lymphocytes were isolated from the spleen and lymph nodes and disassociated into a single cell suspension. After red blood cell lysis, the cells were resuspended to a concentration of $2x10^6$/mL in C10 media (RPMI, with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 uM 2-ME), supplemented with 1 μg/ml OVA peptide (residues 323-329 for DO11.10 Tgs, residues 257-264 for OT-I transgenics). The cells were plated in 24 well plates and incubated for 3 days.

Electroporation of siRNA intro primary T cells

For electroporation, 2.5 μmol dsRNA and/or 20 μg of pEGFP-N3 plasmid (Clontech Laboratories, Palo Alto, CA) were added to prechilled 0.4-cm electrode gap cuvettes (Bio-Rad, Hercules, CA). Activated D011.10 T cells (3 x 10^7) were resuspended to 6 x 10^7 cells/ml in cold serum-free RPMI, added to the cuvettes, mixed, and pulsed once at 310 mV, 975 μF with a Gene Pulser electroporator II (Bio-Rad). Cells were plated into 24-well culture plates containing 1 ml of C10 supplemented with 1 ng/ml IL-2 (BioSource International, Camarillo, CA) and were incubated at 37°C in a humidified 5% CO2 chamber. Cell viability immediately after electroporation was typically around 60%. For cationic lipid transfections, 2 μg of plasmid DNA and 100 nmol siRNAs were used per 10^6 cells, following the manufacturer's transfection protocol. The following siRNA oligos (Dharmacon, Lafayette, CO) were used: CD4 siRNA, (sense)

gagccauaaucucaucugadgdg, (anti-sense) ucagaugagauuauggcucdtdt; CD8 siRNA, (sense) gcuacaacuacuacaugacdtdt, (antisense) gucauguaguaguuguagcdtdt.

Flow cytometry

All wash or resuspension steps were done in FACS buffer (PBS supplemented with 2% FCS and 0.01% sodium azide) or PBS alone. For analysis, cells were washed once and then resupended to 200 μl for cell lines, and 50 μl for primary cells. Primary cells were then stained for 10 min at 4°C with CD16/CD32 antibody to prevent non-specific antibody binding (known as F_c blocking). After blocking, the volume of the primary cells was brought up to 200 ul with PBS. Cells were stained for 20 min on ice with directly conjugated antibodies. After staining, the cells were washed once and resuspended in 100 μl for analysis. Certain stainings also included 500 ng/mL propidium iodide (PI), for exclusion of dead cells. Cell data were collected on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and four-color analyses (Green fluorescence protein (GFP), Phycoerythrin (PE), PI, and Allophycocyanin (APC)) were performed with CellQuest Pro software (BD Biosciences). All data were collected by analyses performed on at least 1 x 10^4 PI-negative events (viable cells), with primary cell analyses performed on at least 2 x 10^4 viable cells.

The following phycoerythrin-conjugated antibodies were used for flow cytometric analysis: CD4 (clone RM4-5), CD8 (clone 53-6.7), CD25 (clone PC81), antibody against CD45.2, CD95.2 (Thy1.2) and streptavidin antibodies. We also used allophycocyanin-conjugated CD4 and CD8, PerCP-conjugated CD4, and biotin-conjugated antibody against Thy1.2 antibodies for analysis. All antibodies were from BD Pharmingen (San

Jose, CA).

RNAi lentivirus system

To generate a lentiviral vector capable of producing shRNAs, the pBFGW plasmid was extensively modified to carry loxP sites, a CMV promoter driving expression of EGFP and the mouse U6 promoter with downstream restriction sites (HpaI and XhoI) to allow the efficient introduction of oligonucleotides encoding shRNAs (see appendix B). ShRNAs were designed using rules from Tuschl and collegues[30]. To these sequences, we added appropriate stem loop, terminator, and restriction site sequences to allow cloning into pLL3.7 (see Figure 1). These longer oligos were 5' phosphorylated and PAGE purified (IDT DNA). After resuspension to 1 ng/μl in water, sense and antisense oligos were annealed together by heating the mixture to 90˚C for 2 min, then 70˚C for 4 min, and then reducing the temperature to 4˚C either at 1˚C/s or as slow as the thermocycler was capable. The annealed oligos were then ligated into HpaI/XhoI digested pLL3.7 using T4 DNA ligase per manufacturer's instructions. Clones were screened via restriction digest and sequence verified. ShRNA sequences used were as follows (targeting sense strand DNA sequence only):

CD8:GCTACAACTACTACATGAC,

CD25:GCATTCACCTAATCGGCTG,

p53:GGTCTAAGTGGAGCCCTTCGAGTGTTA [40],

Mena+:GTCCTGTGCCTGGCCTACT.

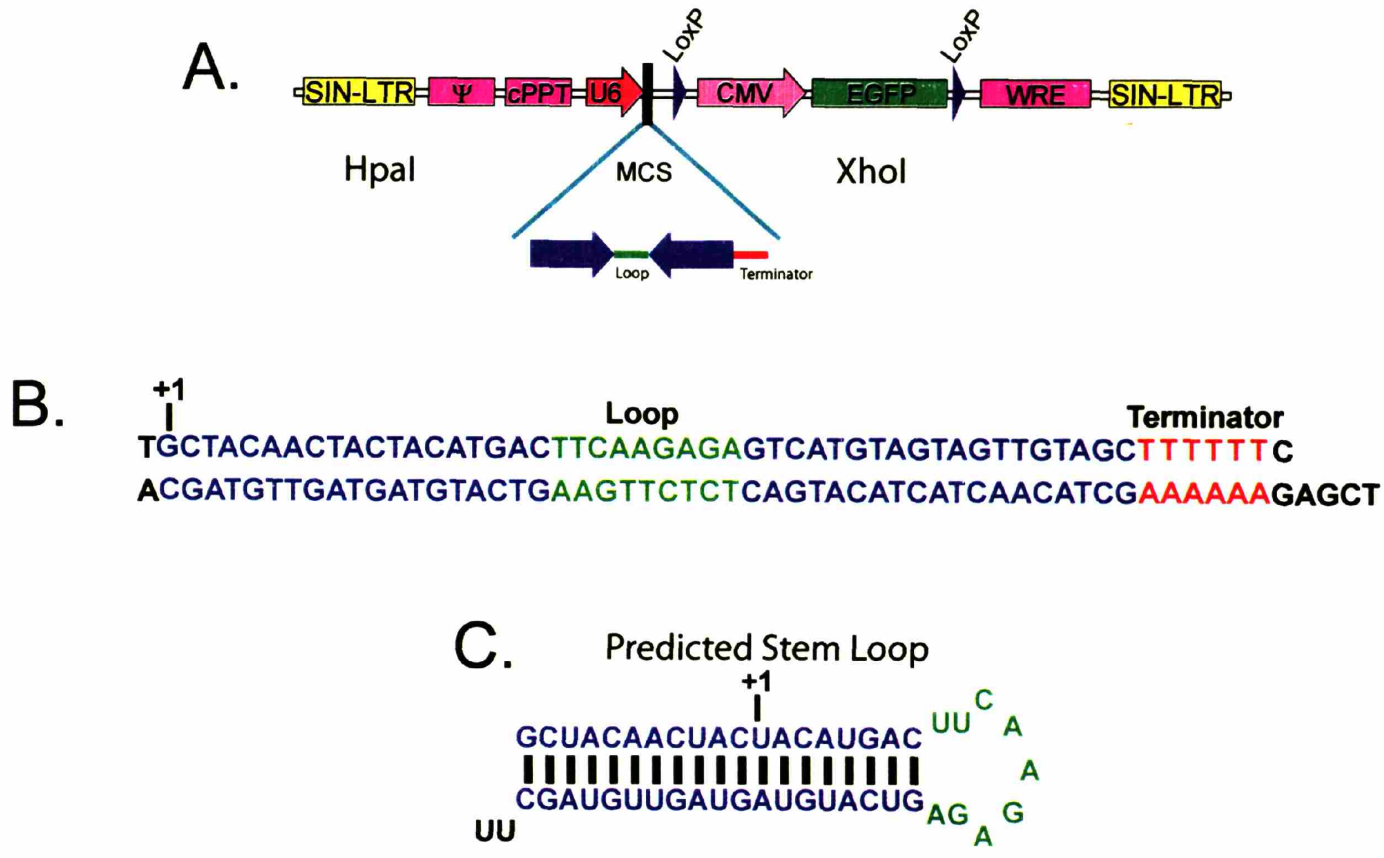


Figure 1. The lentiviral RNAi vector pLentiLox 3.7. **A.** Schematic of pLL3.7. The shRNA is expressed from the U6 promoter, while GFP reporter expression is driven from the CMV promoter. Lox sites flanking the reporter cassette allow it to be excised. HpaI and XhoI are restriction sites for cloning in oligos encoding shRNAs. **B.** Example of DNA oligo sequence for cloning of CD8 shRNA into pLL3.7 vector. **C.** Predicted processed stem loop from pLL3.7 shCD8.

Generation and titration of lentivirus.

We used the 3rd generation packaging system to produced lentivirus. This system consists of the RSV-REV (allows expression of the full viral RNA), pMDLgpRRE (which produces gag and pol), and CMV-VSV-G (for the envelope) plasmids [79]. To produce lentivirus, we co-transfected pLL3.7 and packaging vectors into 293T cells at a 4:2:1:1 ratio using calcium phosphate. We collected the resulting supernatants after 48 and 72 h. We recovered virus after ultracentrifugation for 1.5 h at 25,000 r.p.m. in a Beckman SW28 rotor and resuspended in phosphate-buffered saline (15-200 μl). Titers were determined by infecting 293T cells with serial ten-fold dilutions of concentrated lentivirus. We determined GFP expression of infected cells by flow cytometry 48 h after infection; for a typical preparation, the titer was approximately 4-10 x 10^8 infectious units (IFU) per ml.

T cell infection

CD8-positive T cells were activated by culturing splenocytes derived from OTI TCR transgenic mice at a density of 2 x 10^6 cells/ml in the presence of 1 μg/ml of OVA peptide and 100 ng/ml IL-2 (Biosource International). After 24 h and 48 h, cultures were supplemented with 20-100 x 10^6 lentiviral particles (multiplicity of infection (MOI) of 10-50), 10 μg/ml Polybrene, and spun at 2,500 r.p.m.

ES cell infection

We maintained and infected AK7 ES cells[116]. Briefly, the ES cells were grown on a feeder layer consisting of irradiated embryonic fibroblasts. These were cultured in

the presence of lymphocyte inhibitory factor. The ES cells were placed in single cell suspension and infected with lentivirus overnight in a volume of 500 μl or less. Infected cells were replated and colonies of ES cells were picked, expanded and analyzed by flow cytometry for GFP expression. If the colony contained a mixed population of infected and uninfected cells, we purified the GFP-positive population by fluorescence-activated cell sorting before blastocyst injection.

Generation of RNAi chimeric and transgenic mice

For ES cell-derived mice, we injected approximately 10-12 pLL3.7-infected GFP-positive ES cells into $Rag2^{-/-}$ blastocysts, which we then implanted into a pseudopregnant female recipient mouse. We screened neonates resulting from these injections for chimerism by determining the level of GFP fluorescence of their skin and paws. Highly chimeric (>50%) neonates were used for analysis. To generate lentiviral transgenics, we injected a small volume of high-titre RNAi lentivirus (4-10 x 10^8 IU/ml) into the perivitelline space of single-cell mouse embryos, which we then implanted into pseudopregnant female recipient mice. The resulting neonates were screened for lentiviral integration by Southern blotting and for expression of GFP fluorescence.

Northern-blot analysis

For northern-blot analysis, cells were lysed cells with Trizol reagent (Invitrogen) and total cellular RNA was prepared according to the manufacturer's instructions. RNA was prepared from tissues using RNAlater (Ambion Diagnostics) according to the manufacturer's instructions. We carried out CD4/CD8 probe hybridization[118]. For the

small RNA northern blot, total RNA (60 μg) was fractionated on a 10% denaturing polyacrylamide gel and transferred to a nylon membrane. The membrane was hybridized to a probe consisting of a 21 nt CD8 siRNA sense strand labeled at the 5' end with ^{32}P. We used a 5' radio-labeled oligonucleotide probe to 5S RNA to determine equal loading of RNA.

Western-blot analysis

Mouse tissues were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) supplemented with Complete Protease Inhibitor Tablets (Roche). We determined protein concentrations using BCA Protein Assay (Pierce). Equal amounts of protein (100 μg) were loaded per lane and separated on a 10% SDS-PAGE gel. Protein was transferred to a PVDF membrane. We detected p53 using antibody against p53 Ab-3 (Oncogene Research Products) diluted 1:1,000 and donkey antibody against mouse conjugated with horseradish peroxidase diluted 1:10,000. The blot was developed with ECL+ reagent (Amersham Biosciences).

Results

siRNA-mediated silencing in primary mouse T cells

To test whether mRNA expression in primary immune cells could be silenced using siRNAs, we isolated primary lymphocytes from the spleens of DO11.10 mice. After initially activating the splenocytes with cognate OVA peptide for 72 h, the cells were transfected with siRNAs against the CD4 or the CD8 co-receptor together with a GFP expressing reporter vector. Initially, liposomal transfection reagents were used,

however, neither silencing of the CD4 nor expression of the GFP reporter were seen (Figure 2A). Because of the success of electroporation into T cell lines, we used this method to transfect primary T cells. Despite typically reduced cell viabilities of over 50%, this method was effective for transport of the siRNA into primary T lymphocytes (Figure 2B). CD4 expression was reduced 5 fold and CD8 expression was reduced 3.3 fold compared to controls (Figure 3). Approximately 70% of T lymphocytes in DO11.10 animals are CD4 restricted, allowing the analysis of both CD4 and CD8 expression within the same well. By staining the T cells with antibodies against both CD4 and CD8, we demonstrated that the expression of the alternative CD marker (i.e. CD4 when CD8 was targeted) remained essentially unchanged (Figure 4A). Maximal silencing occurred in these cells at 48 h, after which expression of the targeted receptor began to approach normal levels (Figure 4B).

Creation and Validation of shRNA expressing lentiviral vector LentiLox 3.7

The siRNA experiments in primary T cells showed that RNAi was an efficient process in lymphocytes. However, two major limitations of this method were the toxicity of the treatment and the limited time frame of the silencing. To address these issues, we created a lentiviral vector system capable of expressing shRNAs *in vivo*. We inserted the murine U6 promoter into the GFP expressing LentiLox 3.6 to generate the bifunctional LentiLox 3.7 vector (see appendix for a full description on the design and construction of LentiLox 3.7). To validate this new vector, we adapted the CD8 silencing siRNA from the previous experiment for expression as an shRNA. The vector was tested for efficacy by electroporating both the CD8 shRNA encoding vector and the empty control vector

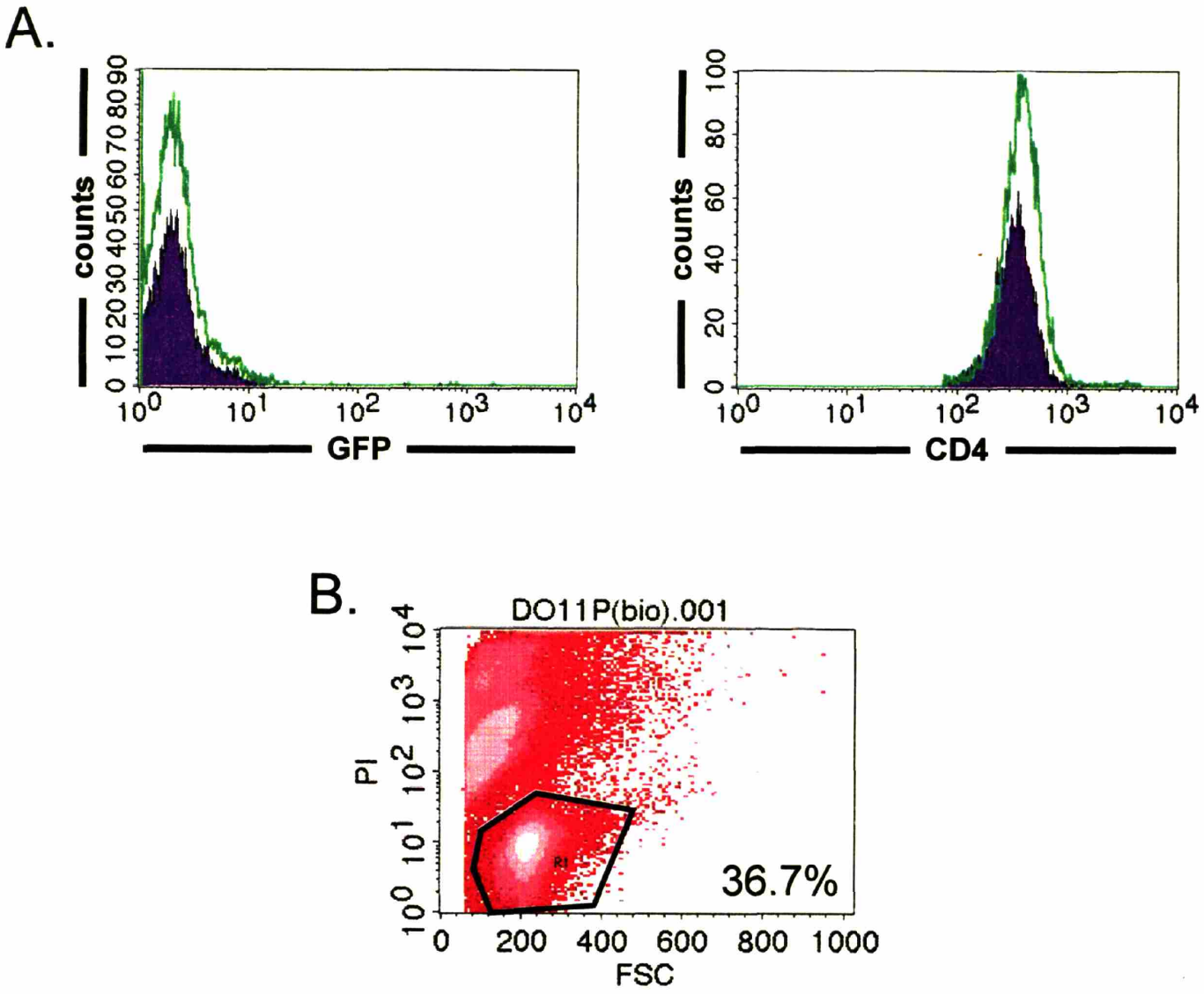


Figure 2. Transfection of siRNAs into primary T cells. **A.** Naïve T cells co-transfected with CD4 siRNA and GFP reporter using Duofect cationic liposomal agent (green lines). Left histogram shows GFP expression and right histogram shows CD4 expression. Non-transfected controls are shown in solid purple. **B.** Activated primary T cells co-transfected with CD4 siRNA and GFP reporter stained with propidium iodide after electroporation to evaluate cell viability. Results from A and B are representative of at least 3 separate experiments.

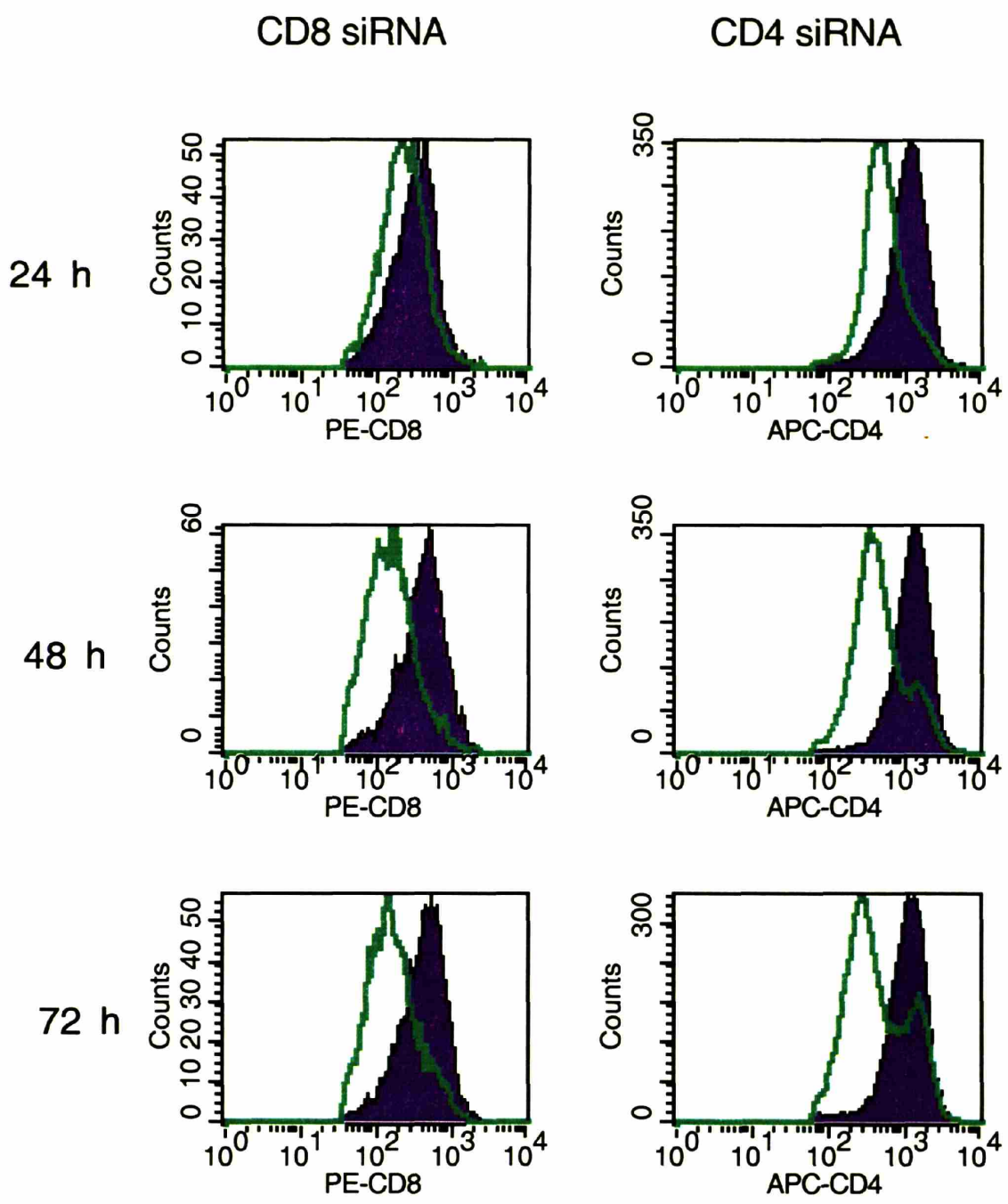
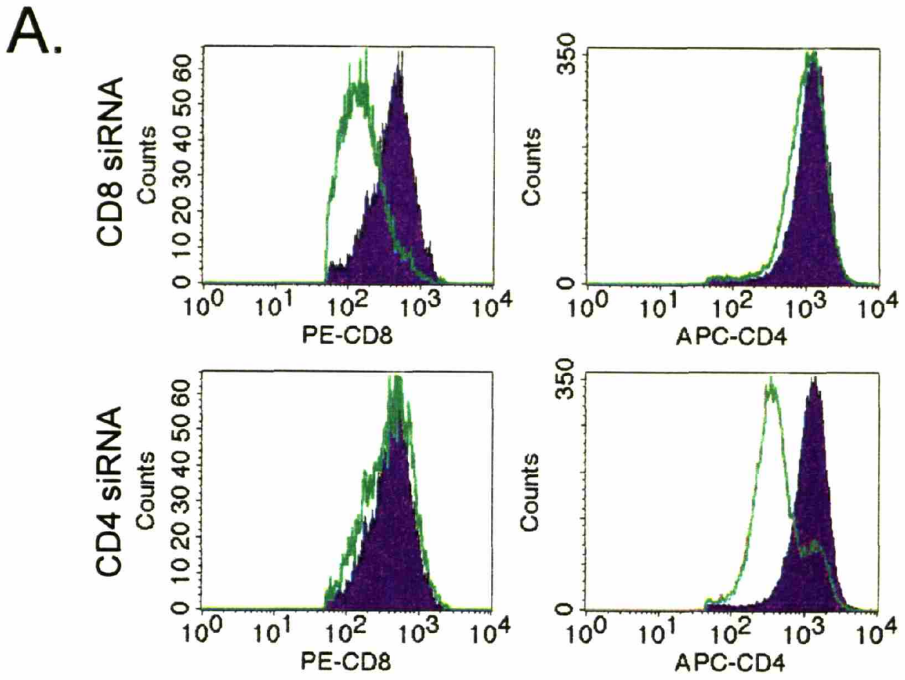


Figure 3. Time course of silencing for activated primary DO11.10 T cells electroporated with siRNAs against either CD8 (left column) or CD4 (right column) (green lines). Solid purple peaks represent cells transfected with a non-specific siRNA control. The histograms are gated on viable cells that express either CD4 or CD8 respectively. Results are representative of 3 individual experiments.



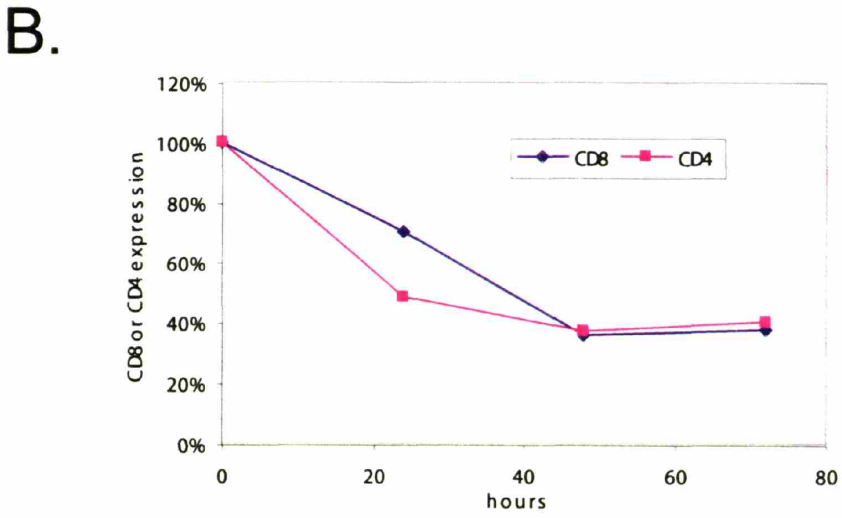


Figure 4. Specificity and time course of siRNA silencing in activated primary T cells. **A.** Activated primary DO11.10 T cells transfected with siRNAs against either CD8)top row) or CD4 (bottom row) stained for both CD8 (left) and CD4 (right) simultaneously to determine specificity of gene silencing. Plots are gated on viable cells expressing either CD4 or CD8. Solid purple peaks are transfected with non-specific controls. **B.** Time course of downregulation of CD4 and CD8 expression in activated primary DO11.10 T cells transfected with siRNAs against CD4 or CD8. The maximal level of silencing was calculated by finding the peak fluorescence level of the suppressed curve and expressing it as a percent of the peak fluorescence level of nonspecific siRNA transfection control. Values are expressed as percent silencing and are plotted against time (hours).

into E10 cells. The E10 T cell line was derived from a thymoma, and thus expresses both the CD4 and CD8 co-receptors. After 48 h, the cells transfected with the CD8 silencing vector showed significant reduction in CD8 levels (Figure 5).

We next tested whether LentiLox 3.7 could silence genes after transduction by infecting E10 T cells with virus produced using 293T cells. E10 cells infected with CD8 targeting lentivirus showed a 94% decrease in CD8 expression with no concomitant change in CD4 levels (Figure 6A). To test whether this silencing was stable, we used a fluorescence activated cell sorter to isolate both GFP expressing control cells and GFP expressing CD8 silenced cells. These cells were grown in culture for over 1 month and then reanalyzed for CD4 and CD8 expression with flow cytometry. The cells maintained expression of the GFP reporter and silencing of CD8 (Figure 6B). Northern blots performed on RNA from these cells showed that while the CD8 transcripts were essentially eliminated, CD4 transcript levels were unaffected (Figure 7A). A small RNA northern showed the presence of a small approximately 21 nt species of RNA corresponding to the sense strand of the shRNA (Figure 7B).

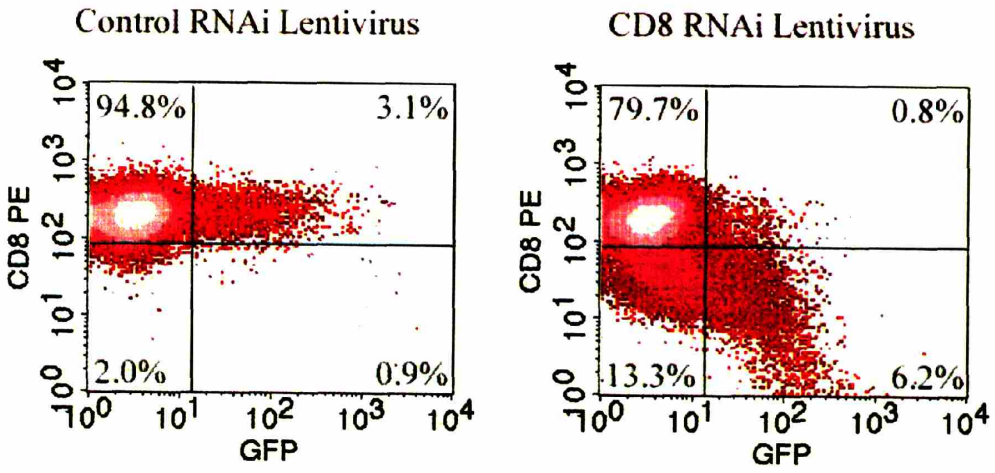


Figure 5. Reduction of CD8 expression in transfected E10 T cells 48 h after electorporation with either pLL3.7 or pLL3.7 shCD8. Plots are gated on viable cells and are representative of two independent experiments.

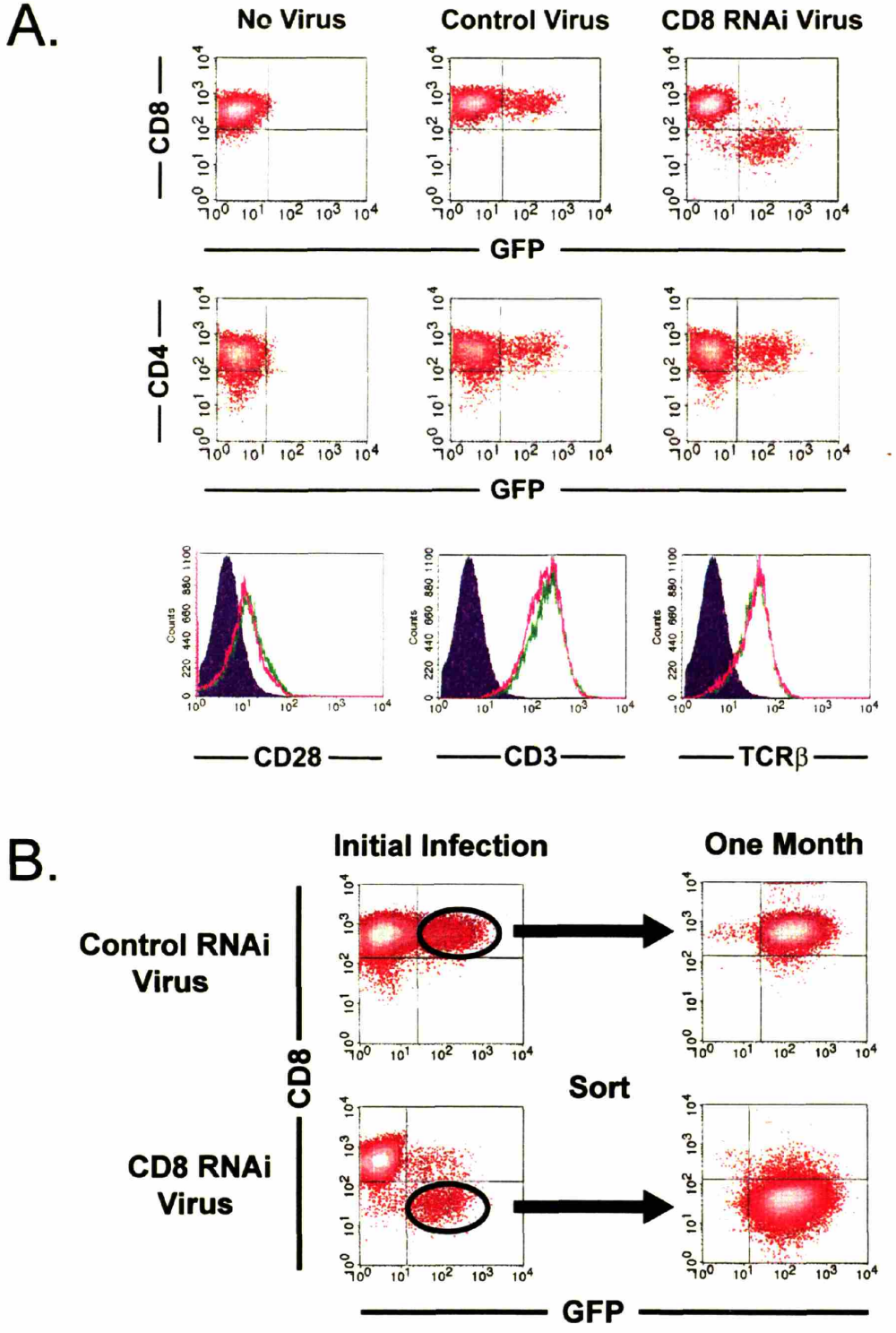


Figure 6. Efficacy, specificity, and stability of silencing by pLL3.7 in a T cell line. **A.** E10 T cells transduced with no virus, pLL3.7 or pLL3.7 shCD8 were evaluated via flow cytometry for CD8 (top) and CD4 (middle row) expression. Bottom row shows staining of other surface markers in GFP positive gated pLL3.7 (green) or pLL3.7 shCD8 (pink) infected E10 cells. Purple peaks are non-stained controls **B.** Stability of CD8 silencing and GFP reporter expression in sorted E10 T cells after one month maintenance in cell culture. E10 cells were transduced with either pLL3.7 or pLL3.7 shCD8 and sorted on the basis of GFP expression and reduced CD8 expression (pLL3.7 shCD8 transduced only).

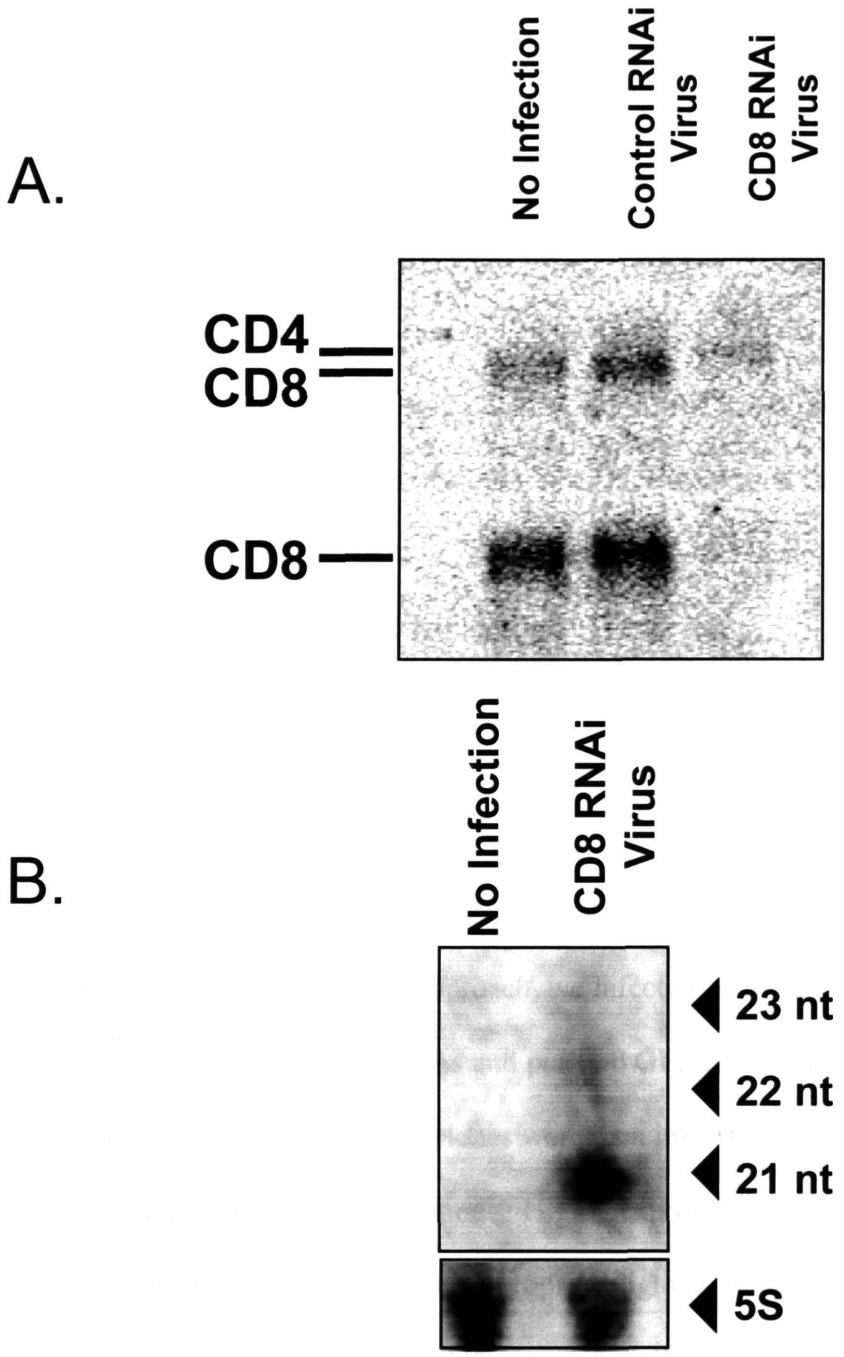


Figure 7. RNA profile of E10 cells transduced with pLL3.7 shCD8. **A**. Specific degradation of CD8 mRNA induced by pLL3.7 CD8. CD8 and CD4 mRNA levels in uninfected E10 cells, or E10 cells infected with either pLL3.7 (control virus) or pLL3.7 CD8 (CD8 RNAi virus) and sorted on the basis of GFP and CD8 expression, were assayed by northern blotting. The bands representing CD8 and CD4 mRNA species are indicated. **B.** Generation of processed shRNAs in cells infected with pLL3.7 CD8. The cells analyzed for CD8 and CD4 mRNA levels in **A** were also examined for the presence of processed shRNAs by northern blotting. The location of RNAs of 21, 22 and 23 nt are indicated.

Verification of lentiviral shRNA efficacy in primary cells

Primary splenocytes from OT-1 mice were isolated and activated with cognate peptide. While undergoing activation, the cells were centrifuged and infected (spinoculated) with control virus, virus targeting CD8, or virus targeting CD25. The cells were analyzed for expression of CD8 and CD25. Infected cells showed a 93% reduction in CD8 expression and a 95% reduction in CD25 expression (Figure 8A). In response to increasing concentrations of IL-2, T cells infected with lentiviruses targeting CD25 showed a 75-80% decrease in proliferation (Figure 8B).

Creating shRNA transgenic animals

Two potential approaches to creating transgenic animals using lentivirus are the injection of infected ES cells into blastocysts and the direct infection of fertilized embryos. To validate the first approach, we infected AK7 ES cells with lentiviruses expressing CD8 targeting shRNAs and purified GFP expressing clones using high speed cell sorting (Figure 9A). These clones were then injected into blastocysts and pups were born from the resulting implantations. Highly expressing chimeras (determined by coat color and GFP fluorescence under appropriate lighting conditions) were euthanized and their organs analyzed via flow cytometry (Figure 9B and C). When compared to controls, the thymi of CD8 knockdown chimeras had an 8.7 fold decrease in mature single positive CD8 cells. In the periphery, the spleen had 6 fold decrease in CD8 cells. Interestingly, the double positive ($CD4^+CD8^+$) subset in the thymus was still present, but shifted compared to controls because of its decreased, but not eliminated, expression of CD8 (Figure 9D).

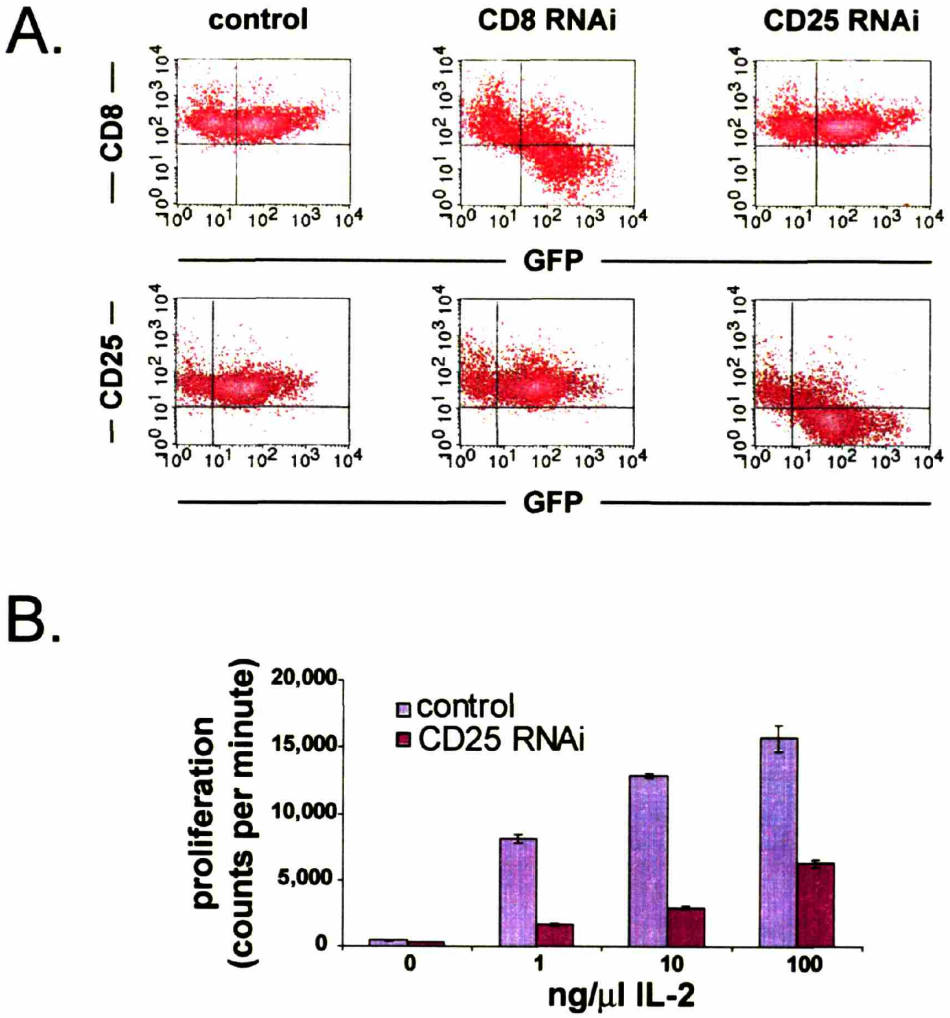


Figure 8. Functional silencing of genes *in vitro* by LentiLox 3.7 in primary T cells. **A.** CD8 (top row) or CD25 (bottom row) expression in non-infected, pLL3.7 shCD8 infected, or pLL3.7 shCD25 infected activated primary OT-1 T cells. The efficiency of infection was determined by assaying GFP expression. Expression of CD8 and CD25 in infected T cells was measured by staining with specific antibodies that bind these surface markers. **B.** Proliferation in response to increasing IL-2 after pLL3.7 or pLL3.7 shCD25 infection of primary $CD8^+$ OT-1 T cells. Proliferation was assessed by ^{3}H-thymidine incorporation done in triplicate wells. Both experiments are representative of two experiments.

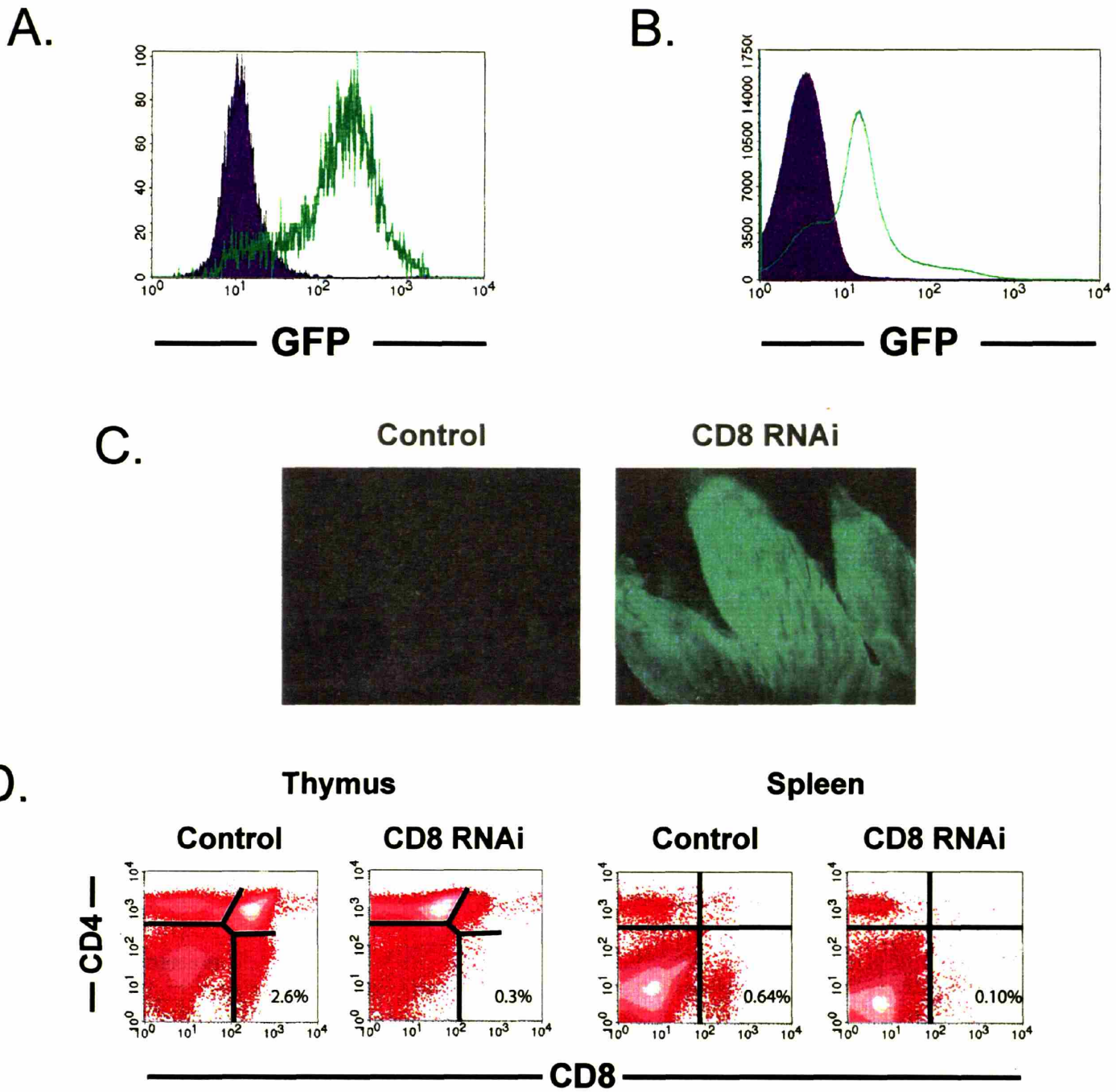


Figure 9. Analysis of ES cell derived RNAi transgenic. **A.** AK7 ES cell line stably infected with pLL3.7 shCD8 (green line) as shown by GFP fluorescence 2 wk after infection compared to uninfected controls (purple peak). **B.** GFP expression in thymocytes from pLL3.7 shCD8 chimeric mouse (green line) compared to thymocytes from non-transgenic littermate controle (purple peak). **C.** Fluorescence imaging of paws of pLL3.7 shCD8 infected ES cell-derived mice using standard epifluorescence to detect expression of EGFP. **D.** Silencing of CD8 in the thymus and spleen of ES cell-derived mice infected with pLL3.7 shCD8. Thymocytes and splenocytes from 1-wk-old control and CD8 RNAi (pLL3.7 shCD8) ES cell_derived mice were harvested and stained for CD4 and CD8 expression. Plots are gated on viable GFP^+ cells.

To validate the second approach for generating lentiviral transgenics, we injected approximately 30 embryos directly with either CD8 targeting or control lentivirus that were then implanted into pseudo-pregnant females. A few embryos in each experiment were incubated *ex vivo* for 3 days and screened for GFP expression via fluorescence microscopy to determine whether the embryos were efficiently infected (Figure 10A). Viable offspring were screened for transgenesis through microscopy or flow cytometry of blood (Figure 10B). Transgenic animals were euthanized and organs from these animals were analyzed via flow cytometry. Northern blots performed on the organ samples showed that the majority of tissues expressed 22 or 23 nt small RNA species corresponding to CD8 shRNA, although these were longer than the 21 nt RNAs found in T cell lines (Figure 10C). Transgenic animals showed more than a 16 fold reduction in the percentage of mature single positive CD8+ cells in the thymus and spleen (Figure 10D).

Discussion

siRNA into primary T cells

These experiments provide the first evidence that primary immune cells are capable of undergoing RNA induced gene silencing. This creates numerous possibilities for immunological study by reducing the time needed to knock out genes of interest. For example, transfecting a 96 well plate of T cells with a different siRNA in each well could be the basis of a genetic screen using *in vitro* readouts such as cell proliferation, cytokine production, and effector function. This could also be a powerful technique for manipulating T cells which are adoptively transferred to other animals. Transfecting

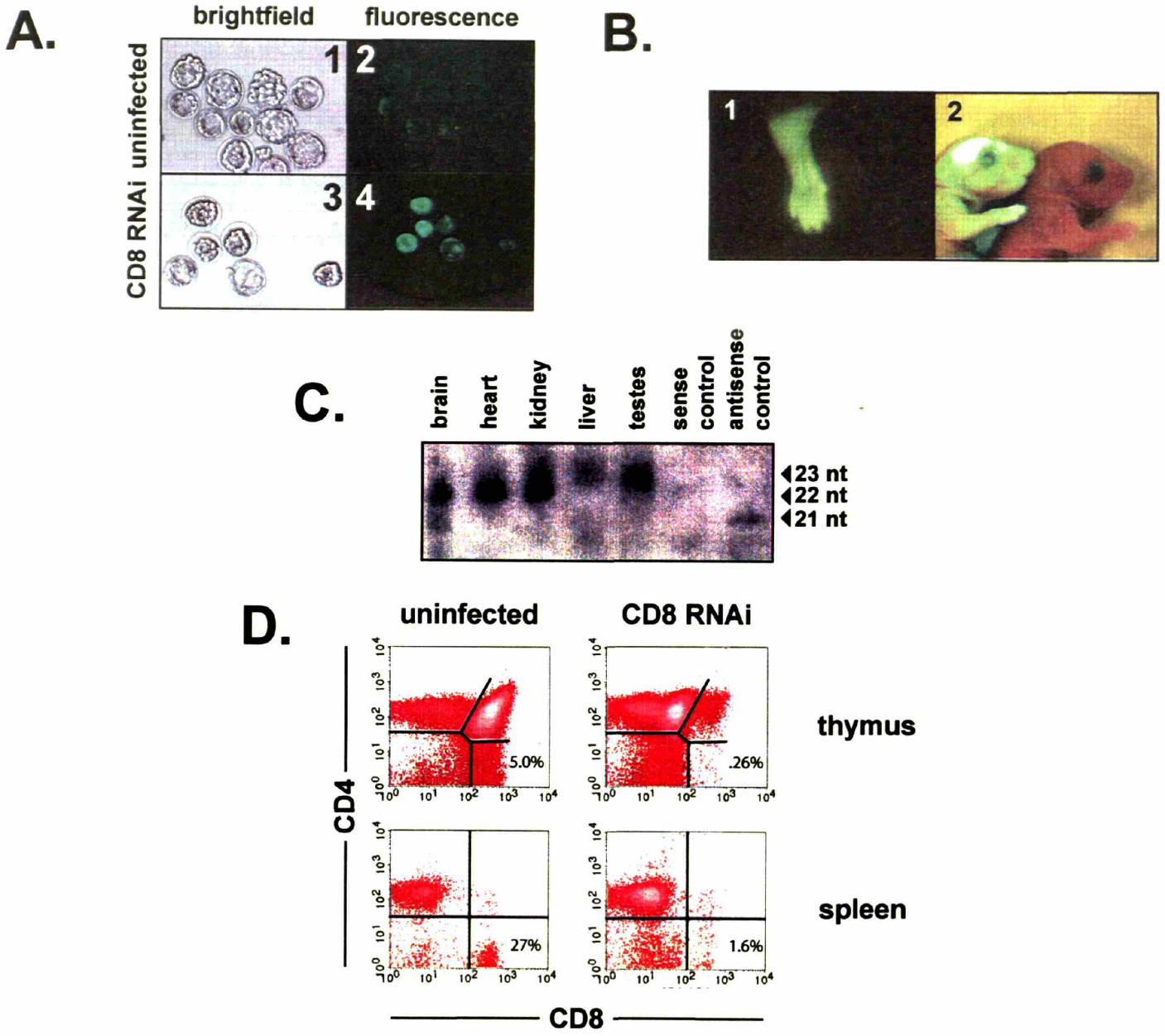


Figure 10. Analysis of embryo derived RNAi transgenics. **A.** GFP expression in embryos incubated *ex vivo* for 3 days after infection with pLL3.7 shCD8. **B.** Fluorescence microscopy of pLL3.7 shCD8 transgenic animals. Left panel is a paw from a transgenic animal, while the right panel is a transgenic pup with its non-infected littermate control. Both panels were imaged with standard epifluorescence for expression of EGFP. **C.** Expression of processed shRNAs in multiple tissues in transgenic mice derived prom pLL3.7 shCD8 infected embryos. Tissues were harvested from 8-wk-old pLL3.7 shCD8 transgenic mice and assayed for the prescence of processed shRNAs by northern blotting. **D.** Silencing of CD8 in the thymus and spleen of transgenic mice derived from pLL3.7 shCD8 infected embryos. Thymocytes and splenocytes from 2-wk-old control and pLL3.7 shCD8 mice were harvested and stained for CD4 and CD8 expression. Plots are gated on GFP^+ cells and are representative of 7 individual pLL3.7 shCD8 animals.

siRNAs could replace the need for maintaining specific knockout strains. Furthermore, electroporation is both safer and easier that using viral delivery systems.

While this method is promising, the protocol contains several challenges to its successful application. Electroporation dramatically reduces cell viability, which can disturb downstream procedures. It is possible to purify viable cells by centrifugation through a Ficoll gradient, but this results in additional cell loss. Improved electroporation technology might circumvent this issue. For instance, a square wave electorporater, such as the BTX instrument, can increase cell viability because the voltage spike is smaller, keeping cells at a transfectable yet less lethal voltage for a longer portion of the pulse. More recently, neighboring labs in the Center for Cancer Research have used the Amaxa nucleofecter to transfect siRNAs into primary T lymphocytes with higher efficiencies and less cell loss.

Transfection into naïve T cells would allow the study of pathways for activation and differentiation into effector cells. My attempts to transfect nucleic acids into these cells proved fruitless. There are at least two explanations for this observation. It is possible that the membrane structure is impervious to transfection, so that the DNA or RNA is not even able to get into the cell. Another possibility is that failure to see silencing is the result of the silencing machinery being inactive in these cells. Naïve cells generally are cells with a small cytoplasm and heavily condensed chromatin, with little metabolic activity, which could partially explain the lack of expression from the reporter vector. The reporter may also fail to be expressed since the vector does not have access to the nucleus for RNA transcription. Transduction of naïve cells by virus has been suggested anecdotally and might circumvent this limitation of siRNA.

The limited duration of silencing could also limit the utility of this technique in primary cells. It might be possible to retransfect siRNA into primary T cell lines to maintain silencing, given the success of this approach in tumor lines. Alternatively, selecting genes with appropriate protein half-life might be important. Genes with extremely fast turnover will be quickly eliminated leaving a short window for experimentation.

Efficacy of the siRNA is also dependent on the efficiency of siRNA transfection, which measured using a co-transfected GFP reporter. Because this is an indirect method, reporter expression may not accurately reflect how much siRNA got into target cells. The use of a plasmid based shRNA expression vector with integrated reporter would allow accurate detection of cells undergoing RNAi.

Lentiviral shRNA vector system

The lentiviral shRNA system was designed to rapidly create transgenic knockdown animals with stable silencing. Our group was the first to generate an RNAi transgenic with an endogenous protein knockdown. The CD8 knockdown altered normal T cell development, indicating that lentiviral induced RNAi will be robust and stable enough to compete with traditional genetic approaches. Less than six months elapsed from the initial cloning and validation of the vector to the analysis of both ES and transgenic animals, which compares favorably to the 1-2 years required to generate a knockout animals.

The practicality of lentiviral RNAi transgenesis might depend on how closely these transgenics phenocopy knockout animals of the same gene. CD8 served as a good

first attempt at answering these concerns. The CD8 co-receptor is encoded by two tandemly arrayed genes, CD8a and CD8b1, which are usually expressed as a heterodimer on T cell surface [119]. Knockouts have been made where either each chain is separately targeted [119, 120], but the resultant phenotypes are grossly similar. In the case of CD8a ablation, neither CD4+CD8+ DP nor CD8+ SP cells are found in the thymus[119]. CD8B is not expressed in the thymus when CD8a has been ablated, suggesting that surface expression of CD8B is dependent on CD8a. In comparison, the CD8B knockout, has CD4+CD8+ DP cells, but these are CD8a restricted and no CD8+ SP cells were present[120]. In neither animal were CD8+ SP cells present in the peripheral lymph organs, such as the lymph node[119, 120]. Our CD8 knockdown targeted CD8a, and yielded animals with a shifted CD4+CD8+ DP population in the thymus, and no normal CD8+ SP in the periphery. Future experiments (see chapter 3, Figure 16), showed that a CD8lo population, where CD8 expression was 1 log lower than normal, was present in the peripheral organs of our lentiviral RNAi transgenics. This was similar to heterozygous knockouts of CD8a where loss of a single copy of CD8 yielded a shifted CD4+CD8+ DP population and allowed development of CD8lo SP cells where CD8 expression was 1/2 to 1 log lower than normal[119]. We did not test whether T cells from our CD8 knockdown transgenics functionally phenocopied the lack of cytotoxic response in homozygous CD8a knockouts or gave a normal response similar to heterozygous CD8a knockouts when exposed to CD8 cell activating antigens such as vaccina. This would have given interesting insight into the functional utility of knockdown transgenics. These observations suggest that for genes where gene dosage is important, the RNAi knockdown might be most similar to the heterozygous knockout, but

it remains unclear if knockdown transgenics might more closely phenocopy the homozygous knockout of genes whose function is not as dependent on gene dosage. Intermediate phenotypes from knockdown animals could provide important insights, especially since, for some molecules, dose response might more accurately mimick human disease.

Transduction of naïve T cells was a goal of using the lentiviral RNAi system instead of siRNAs. Several attempts to infect naïve T cells using our lentiviral system have failed (data not shown), and it remains unclear how permissive of viral infection naïve cells are. Experiments from neighboring labs in the Center for Cancer Research seem to indicate that naïve cells are transduced with lentivirus, but the reporter from that virus does not express until after the cell is at least partially activated (Adam Drake, personal communication). It is unclear from these results whether viral integration occurs before or after activation and further testing will be required to optimize transduction of these cells.

Several elements of the experimental design proved vital for the initial demonstration of lentiviral knockdown animals. The GFP reporter was important for both screening transgenic animals and isolating cells expressing the lentivirus via flow cytometry. Additionally, targeting the cell surface marker CD8 enabled us examine the effects of the knockdown on a single cell level, since neither Western nor Northern blotting can examine protein and mRNA levels in individual cells. This was useful for two reasons. First, at least for CD8, the effect of RNAi appeared to be an all or nothing effect (illustrated in Fig 6). All cells were knocked down to roughly the same level, independent on marker expression. This suggests that even if additional lentiviral

integrants or integration into a more active chromatin region led to higher expression of shRNAs that no further silencing would be achieved. If increased expression of shRNA would have led to greater silencing, we would have expected to see less CD8 expression as cells became more GFP fluorescent. It will be interesting to see if this all or none phenomena holds true for other silenced genes when evaluated on a single cell level. Secondly, we found cells in the FACS analysis that either silenced CD8 or expressed GFP, but not both. Given the close proximity of the pol II and pol III promoters in the integrated provirus, cells that expressed off only one of the promoters likely are victims of competition or interference between the promoters. Fortunately, we found this problem occured in less than 10% of the cells.

While shRNA expression appears robust in many tissues of the transgenic animals, we were unable to determine if the U6 promoter is active enough in all tissues to silence widely expressed genes. Additionally, expression of the shRNA in tissues where our target is not expressed could lead to detrimental off-target effects. Tissue specific or inducible vectors would restrict shRNA expression to targeted regions.

pLL 3.7 shCD8 produced similar knockdown results both *in vitro* and *in vivo*. However, we observed that the small RNA species corresponding to the CD8 shRNA appeared longer *in vivo* than from cells in culture. One possibility is that the specificity of Dicer might vary between mouse strains (the *in vitro* cells were from another strain) or tissues types (the *in vitro* cells are of immune origin), leading to cleavage at a different nt within the step loop. An alternative and more trivial explanation is that the altered migration between tissue and cell lines might result from increased phosphatase activity in certain tissues [P. Sharp, personal communication]. The cleavage of a phosphate group

would slow mobility in the gel towards the cathode compared to an intact RNA of equal length, creating the illusion of a longer species. Cloning and sequencing of the cleavage products would differentiate between these possibilities.

The most exciting implications of this study are those for future investigations where one could use a single vector for *in vitro* and primary cell work, and then use that same vector to create transgenic animals. Before this could occur, the performance of the vector had to be improved. While infected cells showed significant and stable silencing, not every cell in transgenic animals expressed the reporter transgene. There are two possible explanations for this effect: 1) infection occurred after the single cell stage, resulting in a mosaic animal, or 2) an epigenetic silencing mechanism, which could be reset during embryogenesis, was activated in a subset of cells. Even in the breeding lenti-transgenics, GFP expression did not appear in all cells, suggesting that more complex silencing mechanisms are in place.

Chapter 3

Expansion of the lentiviral delivery system

Attribution

Sokol Haxinasto and I generated GM.1 derived transgenics through the MIT Division of Comparative Medicine. We generated, dissected, and analyzed these transgenics as well as the chimeras together. Andrea Ventura and Alex Meissner generated pSICO chimeras on different Cre expressing backgrounds. I dissected and analyzed the immune tissues from these animals via flow cytometry. Patrick Stern cloned the vector with anti-repressors pLB, and Stephan Kissler cloned the EF1a promoter variant pLBEG. Sokol Haxinasto and I used these vector to generate bone marrow chimeras, which I analyzed via flow cytometry.

Introduction

Having demonstrated the value of lentiviruses in generating transgenic RNAi animals, our group was interested in determining how practical and useful generating and analyzing these animals could be for addressing relevant biological questions. We were particularly interested in whether we could restrict transgene expression to a particular tissue, induce shRNA in particular tissues or at a particular time, and reduce the silencing of the lentiviral integrant in animals.

Tissue specific transgene expression

In most early lentiviral transgenic experiments, transgenes were expressed throughout the animal using an ubiquitous promoter such as the ubiquitin, cytomegalovirus (CMV), or phosphoglycerate kinase (pgk) promoter. Many groups, however, are interested in creating vector systems where the transgene is expressed in

only a subset of tissues. Certain polymerase II promoters, when combined with enhancer or silencer elements, restrict their expression to a specific tissue. A number of these promoters, including CD2, lck, CD4, CD19, have been used in the hematopoetic system to generate traditional transgenics. The size of these promoters generally limits their use in lentiviruses since packaging efficiency rapidly decreases as the length of the viral genome increases[121]. Depending on the site of the insertion, every 2kb of additional insert will lead to a 1/2 to 1 log decrease in viral titer [121], because of the reduced efficiency of RNA encapsidation. While some of these promoters are several kilobases long, the CD4 promoter is compact and well characterized [122]. The core CD4 promoter, only 150 bp in length, retains approximately endogenous activity when coupled to a 300 bp enhancer in a viral construct. This minimal promoter/enhancer combination, however, is not exclusively specific for CD4 cells, as it lacks the CD4 silencer to prevent expression within CD8 cells. We possessed a lentivirus using CD4 to drive reporter expression in T cells and we were interested in comparing the specificity of expression between bone marrow chimeras and transgenics. We also examined if the tissue specificity of expression would be reproduced faithfully in progeny.

Inducible shRNA expression

Using the Cre/Lox system, a segment of DNA flanked by Lox sites is eliminated when Cre is expressed. While the Cre/Lox system is frequently used to turn off genes, it can also turn on gene expression when Lox recombination removes a transcriptional stop signal. Since transgenic animals exist where Cre expression is driven by tissue specific or otherwise inducible promoters (i.e. regulated temporally or through drugs), the

Cre/Lox system is an ideal method for regulating gene expression. By breeding a mouse strain containing a lox flanked gene of interest to each of the Cre animals, gene function in multiple tissues and developmental periods could be readily examined.

Collaborators within the Cancer Center used pLentiLox 3.7 as a template for the creation of a Cre-inducible RNAi vector called pSICO [123]. To create an inducible U6 promoter for the expression of shRNAs, the Lox sites were altered to generate a hybrid Lox/TATAbox. The U6 promoter was split by the lox sites, with a CMV-GFP marker cassette in between. Upon expression of Cre, the lox sites recombined, reconstituting a functional U6 promoter enabling hairpin expression. Additionally, reporter expression is lost as a result of the promoter recombination. Having validated the use of the vector *in vitro*, it remained pertinent to further test its efficacy in actuating a biological effect *in vivo*. We generated transgenic animals using a version of pSICO targeting CD8 (pSICO shCD8) to simplify comparison to the original LentiLox experiments. We analyzed pSICO shCD8 animals bred with either Msx2-Cre or Lck-Cre strains to generate the CD8 knockdown.

Improved Vector Expression

While initial experiments demonstrated that lentiviruses can be used to make transgenic and knockdown animals[49, 50], these studies did not examine how penetrant expression was from the lentivirus. Evidence suggests that certain viral vectors, such as LentiLox 3.7 and FUGW, express in no more than 30% of hematopoetic cells [124], even when bred at single viral copy number. Similarly, *in vivo* analysis of pSICO CD8 animals showed that only a portion of cells not induced by Cre actively expressed the

GFP reporter. Thus, there was no way to determine if GFP expression was turned off by Cre expression or if the reporter was never expressed in GFP negative cells. To both increase the percentage of cells expressing the reporter and to stabilize expression across cell types, two DNA elements, AR and SAR, were added to the LentiLox vector to create pLB. AR was originally found in a screen for genomic anti-silencing elements and SAR, characterized by Juergen Bode, maintains open chromatin at sites throughout the genome[125, 126]. To test the efficacy of pLB and a pLB variant using the EF1α promoter, I generated bone marrow chimeras and analyzed reporter expression in blood samples. We also generated and analyzed transgenic animals to evaluate the penetrance of pLB.

Methods

Mice

Several strains of mice were used for these experiments. BL6 and NOD embryos and recipients were used to generate lentiviral transgenics. Hematopoetic stem cells were isolated from either DO11.10 TCR transgenics, BL6, or BL6.SJL (CD45.1) animals, infected and injected into appropriate recipient animals (Balb/C or BL6). Blood samples for screening and analysis were obtained from mice via retro-orbital bleeding. Mice were maintained in specific pathogen free facilities. All the experiments were carried out according to the guidelines established by the Committee on Animal Care at the Massachusetts Institute of Technology.

Flow cytometry

All FACS analysis was prepared as noted in chapter 2, with the following exceptions: some experiments were run on FACScanto 6 color flow cytometer (BD Biosciences) with analysis using FlowJo (TreeStar inc.) and the antibodies listed below were used:

The following phycoerythrin-conjugated antibodies were used for flow cytometric analysis: antibody against CD4 (clone RM4-5), antibody against CD8 (clone 53-6.7), antibody against CD25 (clone PC81), antibody against CD45.2, antibody against CD95.2 (Thy1.2) and strepavidin. We also used allophycocyanin-conjugated antibody against CD4 and CD8, PerCP-conjugated CD4, and biotin-conjugated antibody against Thy1.2 for analysis. All antibodies were from BD Pharmingen.

Generation and titration of lentivirus/Generation of transgenic mice

As described in chapters 2, with the following exception: transfection for viral production used FuGene 6 transfection reagent (Roche Biochem) instead of calcium phosphate.

Lentiviruses

The following lentiviruses were used:GM.1 [122], pLL3.7 shCD8 [50], pSICO, pSICO shCD8 [123], pLB. For a description of the generation of pSICO, see appendix D.

ES cell infection and generating chimeric mice

ES cells were infected with pSICO and pSICO CD8 as described [123]. To

generate animals for analysis, germline chimeras were bred to either Msx2-cre or lck-cre animals. High expressing animals were euthanized and the organs were isolated and prepared for flow cytometry.

Generation of RNAi chimeric and transgenic mice

To generate lentiviral transgenics, we injected a small volume of high-titre RNAi lentivirus (4-10 x 10^8 IU/mL) into the perivitelline space of single-cell mouse embryos, which we then implanted into pseudopregnant female recipient mice. The resulting neonates were screened for lentiviral integration by Southern blotting and expression by GFP fluorescence.

Isolation of hematopoetic stem cells and generation of bone marrow chimeras

Enriched populations of hematopoetic stem cells were isolated by treating donors with 5-fluorouracil (250 μg/g of body weight) 5 days before harvest or by lineage depletion immediately after harvest. Typically, ten to twelve 5-fluorouracil treated animals or 6 non-treated animals were euthanized and the femurs and tibias extracted. Bone marrow was flushed from the bones using PBS blown through 30 gauge needles. Red blood cell lysis was performed on the isolated bone marrow cells. Cells undergoing lineage depletion were pelleted and resuspended in MACS buffer (PBS + 5% fetal bovine serum) and stained with lineage specific antibodies conjugated to streptavidin per manufacturer's instructions (Miltenyi Biotec). After staining, the cells were further incubated with magnetic beads conjugated to biotin. The cells were then washed and suspended in MACS buffer, and isolated using the depletion program in the AutoMACS

machine. Typical recoveries were 10% on input cell number. Cells prepared via either method were plated at 2 x 10^6 cell/mL in 24 well plates in D15 (DMEM with 15% fetal bovine serum) supplemented with cytokines (20 ng/ml IL-3, 50 ng/ml IL-6, and 50 ng/ml stem cell factor). Lentivirus was added to the cells at M.O.I.s ranging from .5 to 5 along with 4 μg/mL polybrene after 24 hours, and the cells were spinoculated for 1 1/2 h at 2500 rpm at 30˚C. After centrifugation, the cells were incubated on the virus for 1 to 6 h, before replacing 70% of the media with fresh media. For injection, cells were washed and suspended in 200 μl of PBS. From 50,000 to 2 x 10^6 cells were injected intravenously into irradiated recipients. Balb/c or Rag -/- recipients were typically irradiated with 900 rad, while BL6 mice typically received 1200 rad. Injected animals were kept on wet food and antibiotics for the first two weeks following reconstituion.

Results

Tissue specific transgene expression

We used the CD4-GFP vector GM.1 to generate transgenic offspring and create chimeric animals by reconstituting the immune system of irradiated recipient animals with infected hematopoetic stem cells. Blood samples from animals derived using both methods were analyzed via flow cytometry. The transgenic animals had a high percentage of GFP-positive CD4 (58.4%) and CD8 (55%) T cells, but very few GFP-positive granulocytes (0.75%) and B cells (2.5%) (Figure 11A). In contrast, GFP was present both in the T cells (45.7% of CD4) and the B cell compartment (37.8%) of the chimeric animals (Figure 11B).

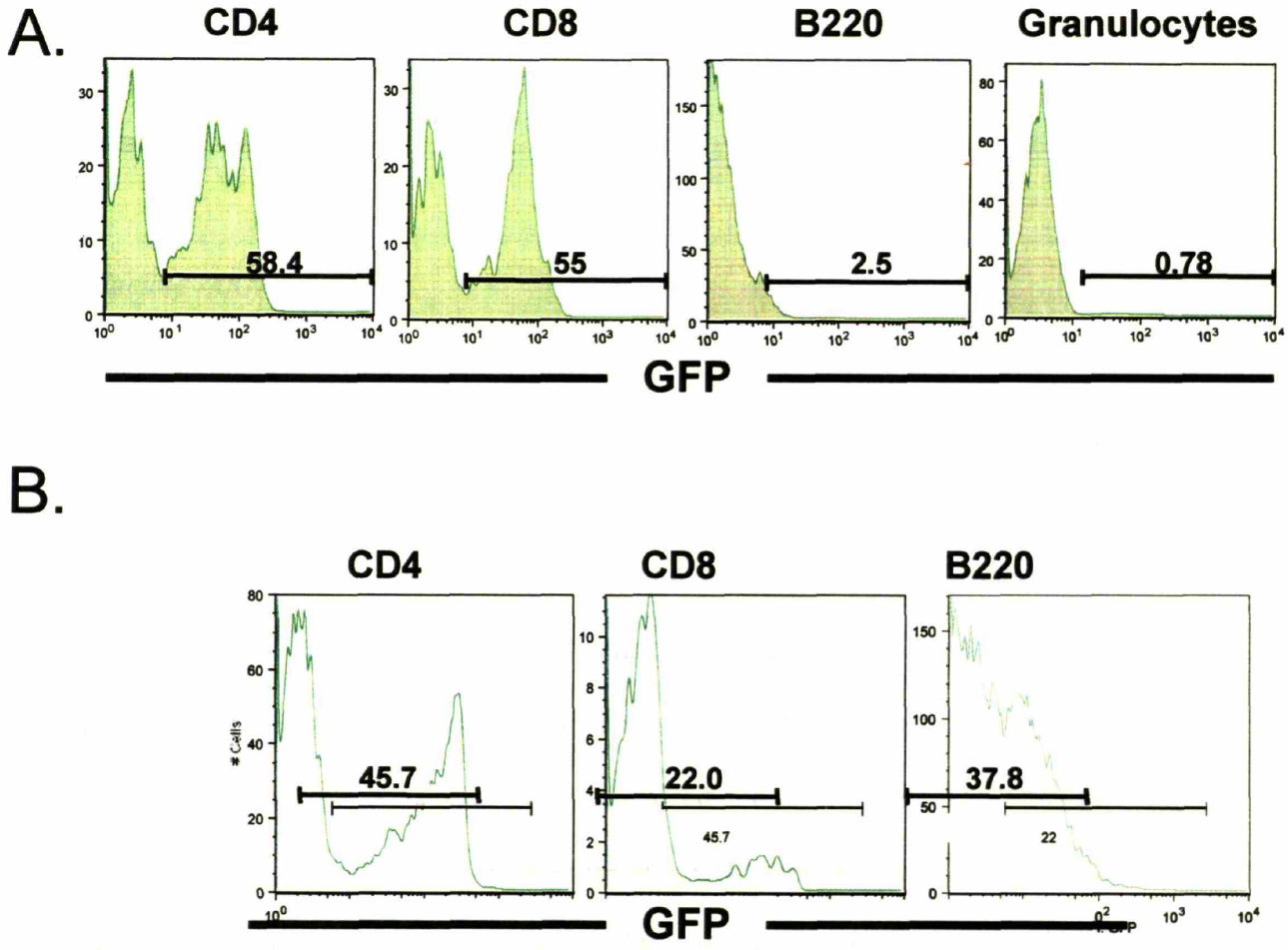


Figure 11. Profiling tissue specific expression in lentiviral transgenics and chimeras derived using CD4-GFP vector. Blood samples were obtained from the animals and stained for CD4, CD8, B220, and CD11b for analysis by flow cytometry. **A.** GFP expression by immune cell type in founder animal derived from GM.1 (CD4-GFP) infected embryos. **B.** GFP expression by cell type in chimeric CD4-GFP animal. Bone marrow was harvested from a C57/BL6 donor treated with 5-fluoruracil to activate and expand the hematopoetic stem cell population. The isolated cells were infected with GM.1 (CD4-GFP) virus and injected into lethally irradiated C57/BL6 hosts. Plots are representative of 7 individual chimeric animals.

We examined how faithful tissue specific GFP expression was in progeny generations by breeding transgenics to wild type mates. A substantial proportion of offspring in each generation had GFP reporter expression (Figure 12). Expression of the GFP reporter increased in T cells (85.8%) in the F1 progeny, and remained stable in the F2 progeny (84.4%) (Figure 13). The F3 offspring reproduced the faithful expression of the other generations (92.5%).

Inducible shRNA vectors

To determine the efficacy of inducible lentiviral shRNA vectors *in vivo*, we derived animals from embryonic stem cells infected with pSICO shCD8. Animals in which the integrant went germline were bred to either Msx2-Cre or Lck-Cre mates. Bleeds were taken from the resulting offspring and analyzed via flow cytometry. These animals had GFP expression levels in B cells ranging from 5-75%, with a high percentage of GFP-negative cells in littermates without CRE present. The offspring with greater than 60% GFP expression were euthanized and their organs were analyzed via flow cytometry. The percent of cells expressing CD8 dropped from 22.6% to 10.3% in pSICO shCD8/Msx2-Cre animals compared to wildtype animals (Figure 14A). In the pSICO shCD8/Lck-Cre animals, the percent of CD8 expressing cells dropped from 33.2% to 15.9% compared to an animal infected with pSICO alone (Figure 14B). There was no substantial difference in the proportion of CD4 between knockdowns and controls (Figure 14A,B). PCR screening showed that recombination was complete in the thymuses of both Msx2-Cre and Lck-cre derived animals.

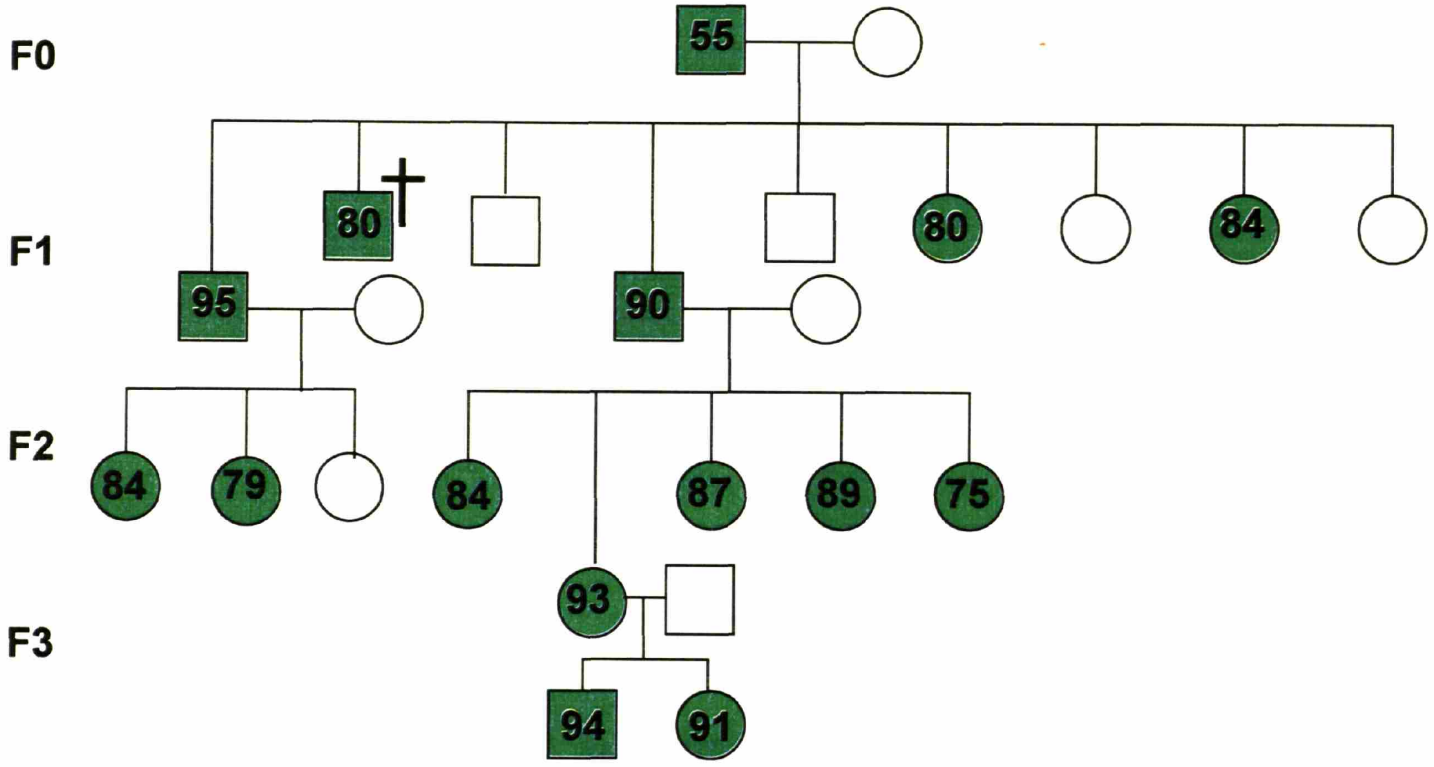


Figure 12. Pedigree of CD4-GFP lentiviral transgenic cohort. A transgenic founder derived from GM.1 (CD4-GFP) infected embryos was crossed to a C57/BL6 female. Offspring were also bred with C57/BL6 mates. Green shading indicates GFP expressing animals as determined by flow cytometry from blood samples. The number inside the box or circle represents expression of GFP in CD8 cells.

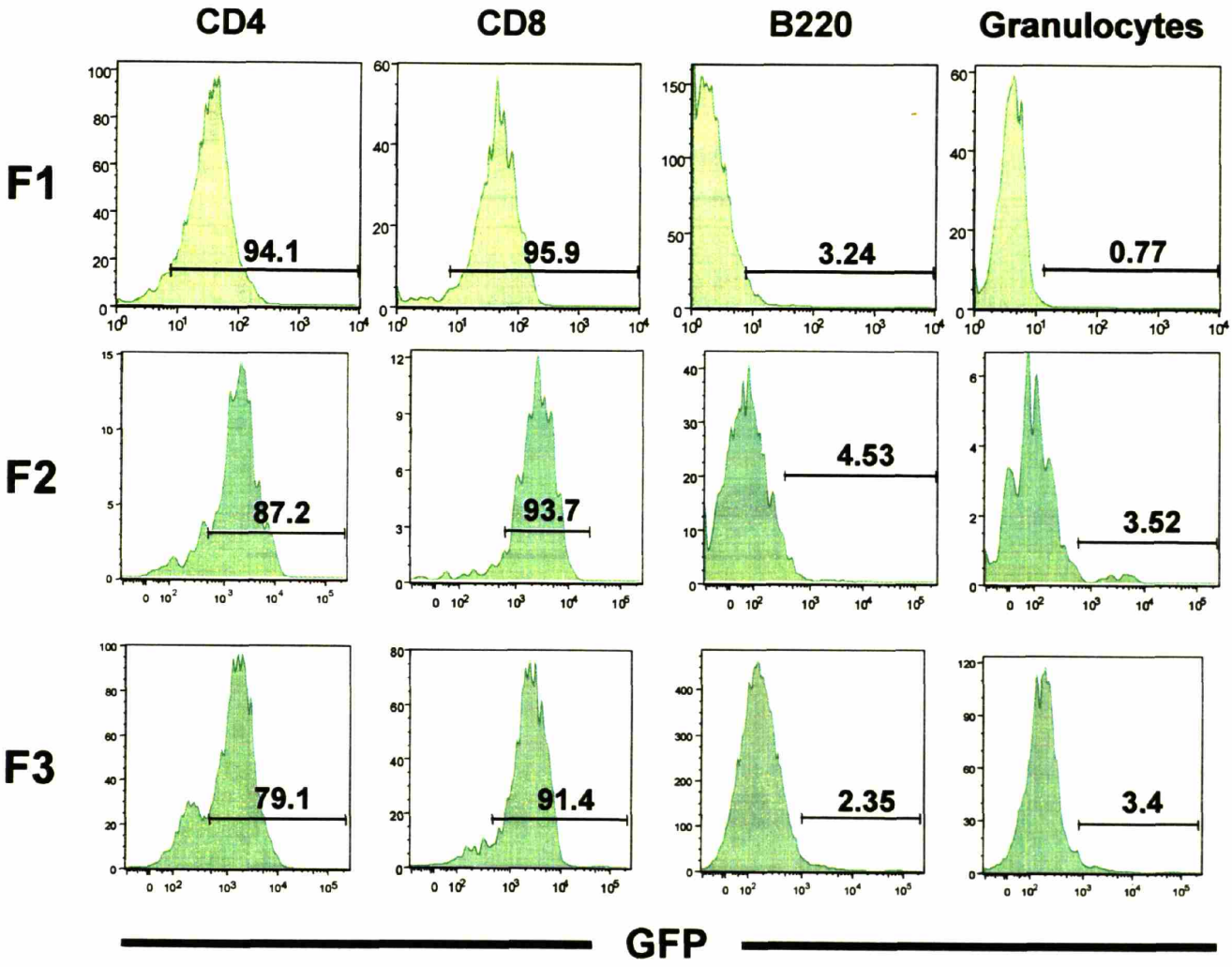


Figure 13. Profiling tissue specific expression in cohort of lentiviral transgenics derived using CD4-GFP vector. Blood samples were obtained from the representative animals of each generation (F1-F3) shown in Figure 12 and stained for CD4, CD8, B220, and CD11b for analysis by flow cytometry.

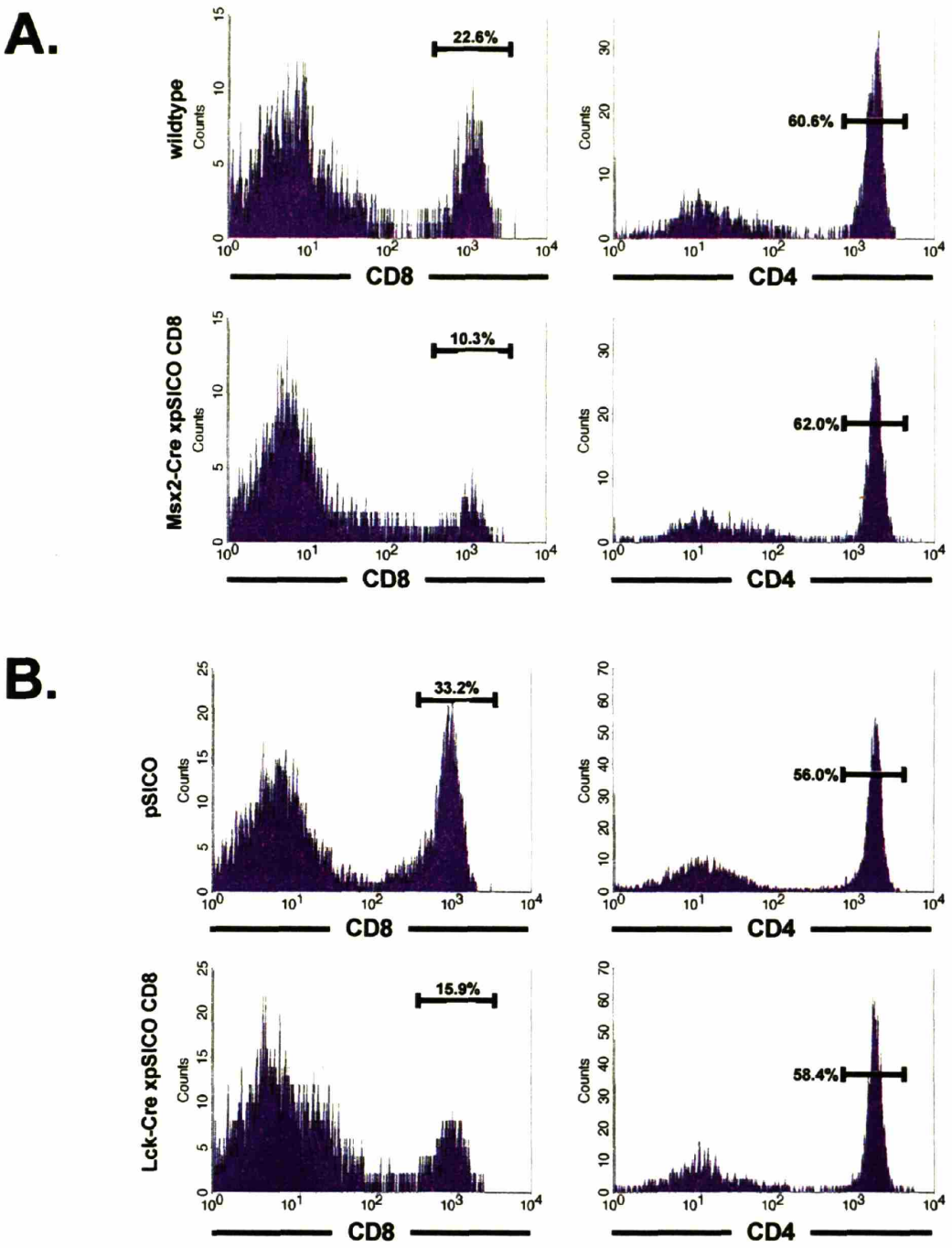


Figure 14. Validation of pSICO vector *in vivo*. Chimeras from pSICO shCD8 infected ES cells were crossed to Msx-2-Cre or Lck-Cre animals. The resulting mice were genotyped for the presence fo Cre and pSICO. Splenocytes from 1- to 3-week old mice with the indicated genotypes were harvested, stained for CD3, CD4, and CD8 expression, and analyzed by flow cytometry. Only $CD3^+$ cells were plotted. One representative example of littermates for each cross is shown. **A.** Splenocytes from control or Msx2-Cre/pSICO shCD8 infected chimeras stained for CD4 and CD8. **B.** Splenocytes from pSICO or Lck-Cre/pSICO shCD8 animals stained for CD4 and CD8.

Improved Vector Expression

We hypothesized that incorporating insulating elements into our vector would prevent silencing and create more consistent transgene expression across tissues. We also tested whether these elements would increase the fluorescence intensity of our reporter, enabling us to more accurately isolate the cell populations expressing the transgene. We compared GFP expression in blood samples from bone marrow chimeras generated using both the original vector and pLB, a vector containing the AR and SAR elements. While the percentage of reporter positive cells remained low, a 1.7 fold increase in mean fluorescence intensity (MFI) was observed in the pLB animals compared to pLL3.7 animals (Figure 15). Thus, we exchanged the CMV promoter, which is often silenced in immune cells, for the EF1α promoter to create pLBEG. Chimeras generated with this vector showed a 4.6 fold increase in MFI compared to pLB chimeras (Figure 15).

We used pLB to generate transgenic animals and compared reporter expression in the immune tissues to that of a control pLL 3.7 shCD8 animal. The pLB animal had higher and more equivalent expression of GFP in all immune cells than the pLL3.7 shCD8 animal (Figure 16).

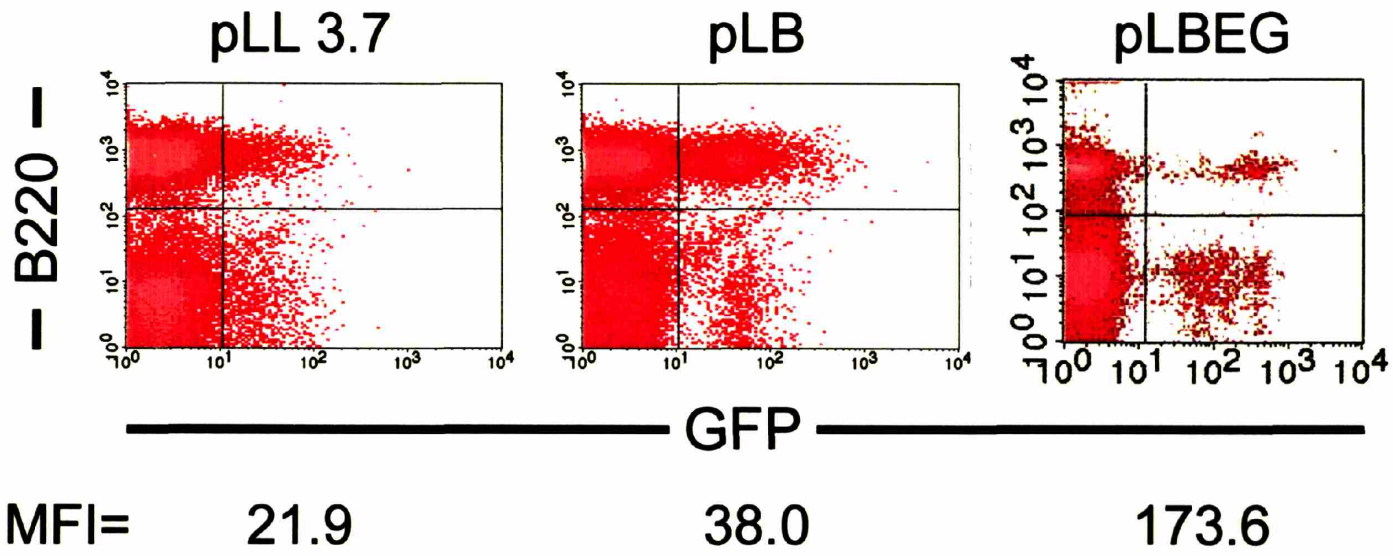


Figure 15. GFP expression from pLL3.7 variants in the B cell compartment of chimeric animals. Bone marrow from C57/BL6 donors was harvested and infected with pLL3.7, pLB (pLL3.7 with anti-repressor elements), or pLBEG (pLB with EF1a promoter replacing CMV). Infected bone marrow was injected into irradiate C57/BL6 recipients. Blood samples drawn 4 wk after reconstitution were stained with B220 and analyzed via flow cytometry. MFI was calculated based on $GFP^{+}B220^{+}$ subset using CellQuest Pro analysis software.

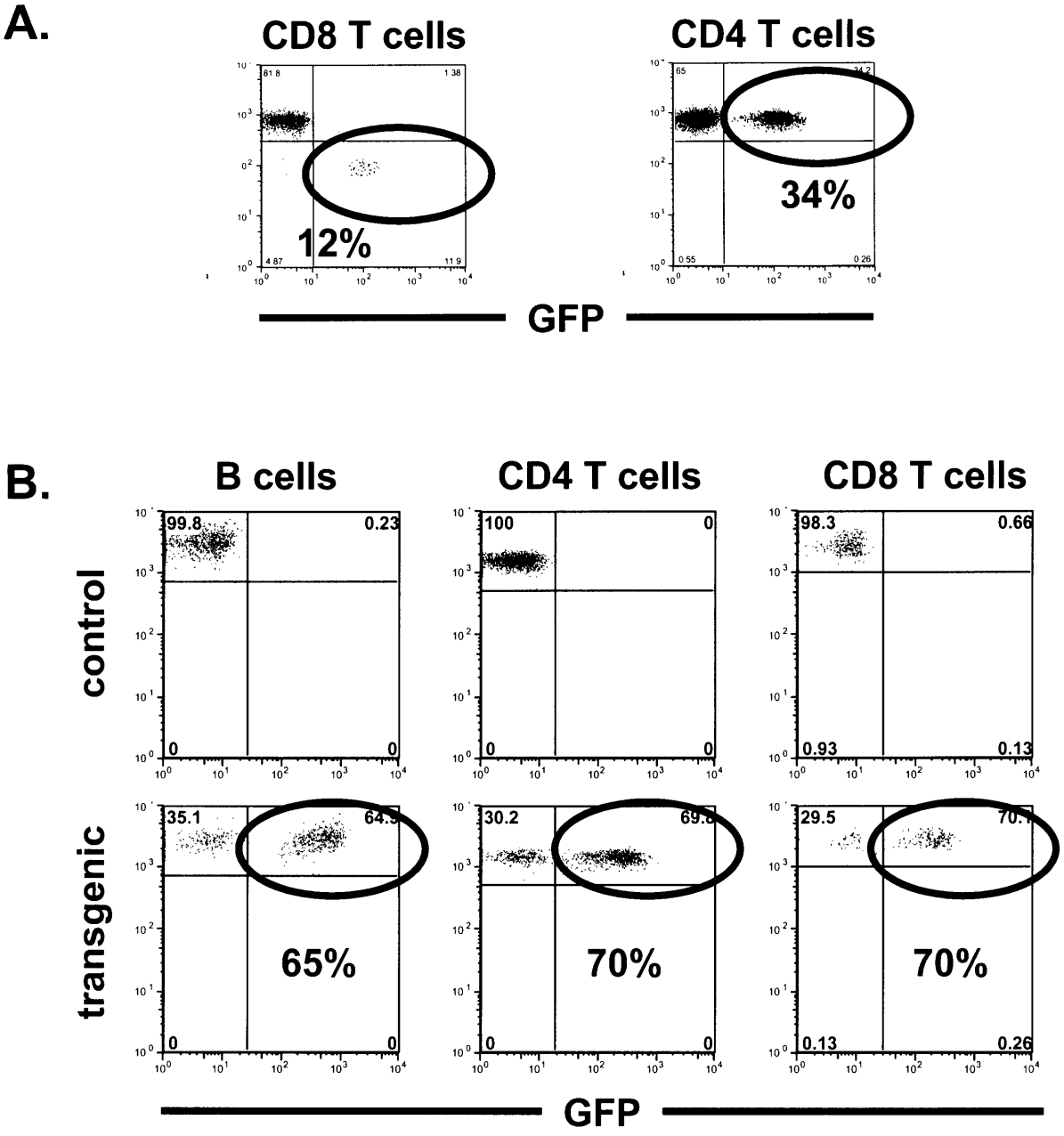


Figure 16. Evaluating the effectiveness of insulator sequences in lentiviral transgenics. Animals were derived from NOD embryos infected with either pLL3.7 shCD8 or pLB. Blood samples from each animal were stained with CD4, CD8, and/or B220 and analyzed via flow cytometry. Cell percentages highlighted were calculated using FlowJo analysis software, **A.** Expression of GFP in pLL3.7 shCD8 NOD transgenic in CD8 and CD4 cells. **B.** Expression of GFP in pLB NOD transgenic in CD4, CD8 and B cells.

Discussion

Tissue specific transgene expression

We used a CD4-GFP lentivirus to achieve tissue specific transgene expression. In transgenics, we found reporter expression only in CD4 and CD8 cells, which was faithful through the F3 generation. This consistency suggests that the large cohorts required for disease studies can be bred, further extending the utility of lentiviral transgenesis. However, a formal evaluation of viral copy number via Southern blot is needed to determine how many viral integrants these animals possess and whether cohorts can be bred from animals that possess a single expressing integrant. Cohorts that utilize only a single integrant are preferred because it simplifies breeding and reduces the likelihood of insertional mutagenesis altering the phenotype. The tails from the CD4-GFP animals were destroyed before Southern blots could be performed on the cohort. This experiment will have to be completed before relying on this or any other lentiviral vector for large-scale disease studies.

While transgenics created using this vector showed T cell restricted reporter expression, chimeras generated using the same vector showed reporter expression in B cells. Since the minimal promoter in the vector contained regulatory elements required to keep expression specific to T cells, expression should have been limited to T cells in the chimera as well. The fact that chimeras showed expression in B cells suggests that there may be other regulatory sequences important for T cell specific expression that are deactivated once more permanent forms of repression, such as methylation, are established. Expression from our vector in the chimeras might escape this suppression because the viral integrant was not present when methylation was initiated. In

transgenics, the integrated virus is present earlier in development when this methylation takes place. While other groups have reported that chimeras generated with this vector are T-cell restricted, closer evaluation of their data shows that at least some of their chimeras had significant reporter expression in B cells [122]. Another possible explanation for the observed loss of specificity in the chimera is the random integration of the lentivirus into active loci. Read-through from an endogenous promoter through the integrated virus might result in ectopic expression in non-T cells. This problem would be limited in transgenics, because many of the genes that are active when the embryo is infected are turned off in the developed animal. Thus, further research will be required before the specificity and general utility of this vector for making bone marrow chimeras can be determined.

Inducible shRNA expression

An inducible vector enabling tissue or temporal specific expression could catalyze research in many areas of biology. When we validated use of the inducible vector pSICO *in vivo*, we observed variability in expression of the reporter gene. One explanation for this could be mosaicism resulting from both infected and non-infected ES cells contributing to the chimera. . The ES colony selected for injection was generated by visually selecting infected cells from colonies after infection. The animals were generated by injecting 10-12 pSICO infected V6.5 ES cells into recipient blastocysts. While neither procedure guarantees a clonal ES population, the resultant animals were screened for high levels of chimerism and bred out against wild-type or Cre expressing animals. Even if the parental chimeras were mosaic, each sperm from these animals must

be derived from a single cell source and cannot be mosaic. Hence, the variability likely resulted from silencing of the viral vector.

In most of the animals analyzed, GFP was expressed in only a portion of the cells not exposed to Cre, making it unclear if GFP-negative cells were expressing the shRNA. To address this concern, we analyzed animals with a high GFP expression in control tissues. Even in animals with low GFP expression (under 25%), Cre mediated recombination appeared complete. Thus, it was not clear whether shRNAs were being expressed in the cells after recombination even when no GFP was expressed before recombination.

Problem with reporter expression will probably reduce the effectiveness of pSICO for generating bone marrow chimeras. The chances of achieving expression in every cell is reduced in bone marrow chimeras, since they are generated from a polyclonal cell population, unlike transgenics developed from an infected single cell embryo. Enriching for infected hematopoetic stem cells by sorting before injection into recipients might create animals with more pure expression. Another promising solution is to add a second marker that will report as long as the whole integrant is active. For example, the fluorescent reporter RFP, which is distinguishable from GFP via flow cytometry, could be added. However, the additional insert of approximately 1.5 kb for the promoter and second marker gene could result in a 5 to 10 fold decrease in titer, potentially making bone marrow experiments impractical [121]. In addition, more significant problems with promoter interference could occur. Further work regarding these issues could make pSICO a viable tool for research into the immune system.

Improved Vector Expression

We tried to increase the efficiency of the lentiviral vector system by adding insulating elements to the vector. We used the pLB vector, which included two insulating elements, to generate bone marrow chimeras, resulting in higher MFI in marker positive cells and enabling clear differentiation between positive and negative cells. An even higher MFI was found in positive cells using a pLB variant containing EF1α promoter. These initial experiments suggest that our modifications of pLL3.7 resulted in vectors with higher quality expression. Since the injected bone marrow had a mixed population of viral integrants, these experiments did not show whether the additional elements helped reduce silencing. Therefore, we made transgenics from infected single cell embryos to test the silencing of these improved vectors. The transgenics had increased reporter expression compared to normal LentiLox derived animals. The improved vector also equalized reporter expression in various hematopoetic tissues such as B and T cells. Despite vector improvements, not all the cells in the transgenic animals expressed the reporter.

Mice have developed mechanisms to silence endogenous retroviruses, and it has been suggested that they may have evolved a similar mechanism to silence lentiviruses. This seems unlikely, however, since there are no endogenous murine lentiviruses, and thus little evolutionary pressure to create defense mechanisms against them. In addition, it would not have been possible to generate adult transgenic animals using lentivirus if specific silencing mechanisms existed. Other mechanisms must therefore result in lentiviral silencing. Since lentiviruses tend to integrate into active promoter regions, it is possible that the integrated provirus became silenced in certain cell types as the embryos

differentiated and open loci were shut off. Stochastic mechanisms, an extension of silencing inherent to normal cell function, might also silence the integrant in a certain percentage of the cells. General silencing mechanisms against viral elements, such as repetitive sequences in the LTR or transcription factor binding sites in the 3'UTR, could also occur. Elimination of these sites from the vector might reduce the level of silencing currently observed. Additional testing of the pLB system is needed to determine if expression can be further increased. Nevertheless, this method is useful for experiments that do not require complete ablation of the targeted protein, such as investigations of cell autonomous processes.

Chapter 4

Evaluating effector caspase function via RNAi

Attribution

I designed and cloned the caspase shRNA vectors together with the assistance of my undergraduate student Nathan Liu. Some of the shRNA sequences I designed were outsourced for cloning, along with other sequences from the lab. Andreas Bonertz, whose data is included in this thesis, used my caspase vectors to help validate Peter Sandy's luciferase shRNA validation system (Figure 18). Sokol Haxinasto gave me technical assistance in generating LentiLox chimeras, which I analyzed via flow cytometry. I performed all the apoptosis and Western blot experiments in this chapter.

Introduction

Caspases are integral components of the programmed cell death, or apoptotic, pathway, serving to couple upstream death inducing signals with downstream phenotypic manifestations of apoptosis [127]. Apoptosis is an important process in the adaptive immune system, helping to eliminate excess target-specific cells after an immune response and self-reactive cells during immune development [128]. Knockouts of certain caspases lead to embryonic and perinatal lethality, indicating that these genes and the processes they mediate play an important role in development [106]. Considerable effort has been extended to examine the specific role of caspases in immune cell apoptosis.

Apoptosis is important in the life cycle of immune cells at two distinct points. Apoptosis shapes the development of the T-cell repertoire, the collective specificity of all the T-cell receptors in an individual's immune system [129]. Receptor specificity is determined in the thymus during T cell development [129]. Cells are eliminated if they do not express a functional T-cell receptor, express a receptor which doesn't recognize

the antigen presenting molecule MHC (lack of positive selection), or if the receptor strongly recognizes self-protein (negative selection) [130]. After T-cell development, apoptosis is essential for the elimination of excess antigen-specific T cells whose population expanded to eliminate the threatening insult [131].

Caspase–9 is a key initiator caspase whose role in immune system function has been difficult to examine since knockouts survive only to the end of gestation [103, 104]. Bone marrow chimeras derived from injecting casp9-/- fetal livers into irradiated recipient animals have been used to evaluate the role of caspase-9 in immune cells [132]. The thymal profile from casp9-/- chimeric mice looks similar to wild-type, suggesting that caspase-9 does not play a role in negative selection [128, 132]. Caspase-9 is unnecessary for peripheral T-cell death induced by cytokine withdrawal or dexamethasone [115]. However, casp9-/- thymocytes are resistant to dexamethasone and γ-irradiation induced cell death [103]. Despite playing a central role in apoptotic pathways induced through the mitochondria, caspase-9 ablation appears to have little effect on T cell death. It has been proposed that caspase-7 might compensate for the absence of caspase-9 [115]

The role of caspase-8 in T-cell development has been more difficult to determine, since caspase-8 knockouts are embryonic lethal [110]. However, caspase-8 ablated clones of human Jurkat T-cells are completely resistant to death receptor mediated cell death (i.e. Fas-induced death), and partially resistant to other inducers of cell death (such as etoposide or irradiation) [133]. In animals where caspase-8 was ablated specifically in T cells, thymocytes and peripheral T cells were resistant to Fas-induced cell death [111]. However, there were a reduced number of T cells in the spleen, and *in vitro* cells failed to

activate as well as controls cells [111]. These defects result because caspase-8 is required for activation of NF-kB downstream of the T cell receptor [134]. Thus, caspase-8 plays important roles for both the establishment and downregulation of the immune response.

The effector caspases, caspases-3,-6, and –7, are highly similar in sequence. Caspase-3 and caspase-7 are the most closely related with 55% identity (see Table 1) [135]. Caspase knockouts have only begun to address the functions of these closely related caspases. Caspase-3 is the dominant effector caspase, has the broadest range of cleavable targets, and caspase-3 knockouts are the best characterized of the effector caspases [106, 136]. Despite the fact that caspase-3-/- thymocytes undergo normal apoptosis in response to death stimuli as diverse as Fas, dexamethasone, or γ-irradiation, these cells do not show characteristic apoptotic phenotypes such as membrane blebbing and nuclear fragmentation [137]. In the periphery, mature caspase-3-/- T cells are resistant to death induced by Fas or through the T cell receptor [138]. Rather than a defect in apoptosis, caspase-3 knockouts animals have lymphadenopathy and splenomegaly resulting from increased proliferation of B and T cells as compared to wild-type controls [139]. Because the lack of caspase-3 disrupts p21 binding to PCNA, allowing recruitment of active CDKs, B cells from knockout animals do not regulate cell cycle properly [139].

Given its high degree of identity with caspase-3, caspase-7 would be expected to have similar functions. Since the caspase-7 knockouts are embryonic lethal, it has been difficult to determine its role in the immune system [106]. *In vitro* experiments using chicken DT-40 B cells confirmed earlier findings in murine WEHI B cells that caspase-7

	Similarity		
	Casp6	**Casp3**	**Casp7**
Casp7	57.72	69.26	
Casp3	58.32		54.88
Casp6		38.27	39.43
		Identity	

Table 1. Homology of effector caspases. Adapted from Juan et. al, *Genomics*, 1997. "Percentages of similarity (top, light grey) and identity (bottom, dark grey) obtained by comparing two polypeptide sequences using the BESTFIT program (Genetics Computer Group) at shown."

is involved in B cell death [140, 141]. Treating the WEHI cells with antibodies against the B-cell receptor, a model of immature B cells undergoing selection, results in caspase-7 activation [141]. The role of caspase-6, which is highly similar to both caspases-3 and –7, in the immune system has also been difficult to elucidate. While the caspase-6 knockout shows no overt phenotypes, no systematic evaluation of their immune system status or function has been published [106]. Experiments on human B cells suggest that caspase-6 is required for the regulations of cyclins, which are required to enter the cell cycle [142].

While knockout and *in vitro* studies have provided some insights, the specific role of each effector caspase in the immune system remains unclear. *In vitro* studies show a high overlap in cleavage specificity between caspase-3 and –7 [143]. The role of caspases -6 and –7 is unclear as caspase-3 is required for almost all important cleavage events for the execution of apoptosis [136]. Evidence suggests that caspase-7 compensates for caspase-3 during brain development in the C57/BL6 background [113], suggesting that these caspase have overlapping roles. However, the roles of all the caspases involved in apoptosis has not been evaluated in a single cell line, so researchers cannot directly compare their functions. To enable such a comparison, I generated lentiviral RNAi vectors targeting each of the effector caspases and used these to generate cell lines ablated for individual or combinations of caspases. In addition, I used lentiviral RNAi vectors to create bone marrow chimeras to evaluate the function of caspse-7 in immune development.

Methods

Mice

Several strains of mice were used for these experiments. BL6 embryos and recipients were used to generate lentiviral transgenics. Hematopoetic stem cells were isolated from either DO11.10 TCR transgenics, BL6, or BL6.SJL (CD45.1) animals, infected, and then injected into appropriate recipient animals (Balb/C, BL6, or BL6/Rag2-/-). Blood samples for screening and analysis were obtained from mice via retro-orbital bleeding. Mice were maintained in specific pathogen free facilities. All the experiments were carried out according to the guidelines established by the Committee on Animal Care at the Massachusetts Institute of Technology.

Cell culture

DO11.10 is a murine hybridioma line derived from a fusion of cOVA specific T cells with an AKR hyridoma line[144]. These cells, which proliferate vigorously, were maintained at a maximal concentration of 1 x 10^6 cells/ml and were propagated in C10 media (RPMI, with 10% heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME).

Flow cytometry

All wash or resuspension steps used FACS buffer (PBS supplemented with 2% FCS and 0.01% sodium azide) or PBS alone. For apoptotic assays, cells were washed once and resuspended in 100 μl of a 500ng/mL propidium iodide solution. At least 10^4 total

events were captured during apoptosis assays. All other FACS analysis was prepared as noted in chapter 3.

The following phycoerythrin-conjugated antibodies were used for flow cytometric analysis: antibody against CD4 (clone RM4-5), antibody against CD8 (clone 53-6.7), antibody against CD25 (clone PC81), antibody against CD45.2, antibody against CD95.2 (Thy1.2), and strepavidin. We also used allophycocyanin-conjugated antibody against CD4 and CD8, PerCP-conjugated CD4, and biotin-conjugated antibody against Thy1.2 for analysis. All antibodies were from BD Pharmingen.

Caspase shRNA design and validation

Oligos specific for the knockdown of each of the caspases were designed either according to the original Tuschl rules or the revised Reynolds rules [30, 33]. These oligos were ordered after short sequence blasting and then cloned into LentiLox 3.7. The caspase vectors were then transfected into 293T cells and virus was harvested after 48 h and used to infect the DO11.10 T cell line. High speed cell sorting was used to isolate polyclonal populations in which all cells were infected, as determined by GFP expression. Protein and RNA were extracted from these sorted cells. Protein extracts were used for Western Blot analysis. Either RT-PCR or luciferase assays were used to determine if caspase mRNAs had been repressed. Sequences are listed in Appendix E.

Generation and titration of lentivirus/Generation of transgenic mice/Generation of bone marrow chimeras

As described in chapters 2 and 3, with the following exception: transfection for viral

production used FuGene 6 (Roche Biochemicals) instead of calcium phosphate.

Western-blot analysis

As performed in chapter 2 with the following changes: caspases-3,-6,-7, and –9 were detected using rabbit antibodies (Cell Signalling) diluted 1:1,000 and goat antibody against rabbit conjugated with horseradish peroxidase diluted 1:5,000. The blot was developed with ECL+ reagent (Amersham Biosciences). Blots against murine tubulin or gamma globulin were used to control for protein loading.

Results

Design and validation of caspase oligos

I generated pLL3.7 shRNA vectors against each of the murine caspases and infected DO11.10 T cells (sequences listed in Appendix E). Protein and RNA samples were isolated from infected cells and analyzed for caspase expression (Figures 17 and 18). A summary of the protein and RNA results is shown in Table 1. Knockdowns were determined to be effective if there was at least a 50% knockdown at the protein or RNA level, which facilitated comparison to rates of designing effective shRNAs in the literature. Interestingly, several of the caspases were easy to target, with only 1-3 distinct sequences tested before a highly effective sequence was discovered. Others, like caspase 8, initially lacked a highly effective shRNA sequence despite testing of over 7 distinct silencing sequences, including several reported as effective in the literature. Effective caspase-8 shRNAs were later designed with the use of the Reynolds algorithim (Figure 18) [33].

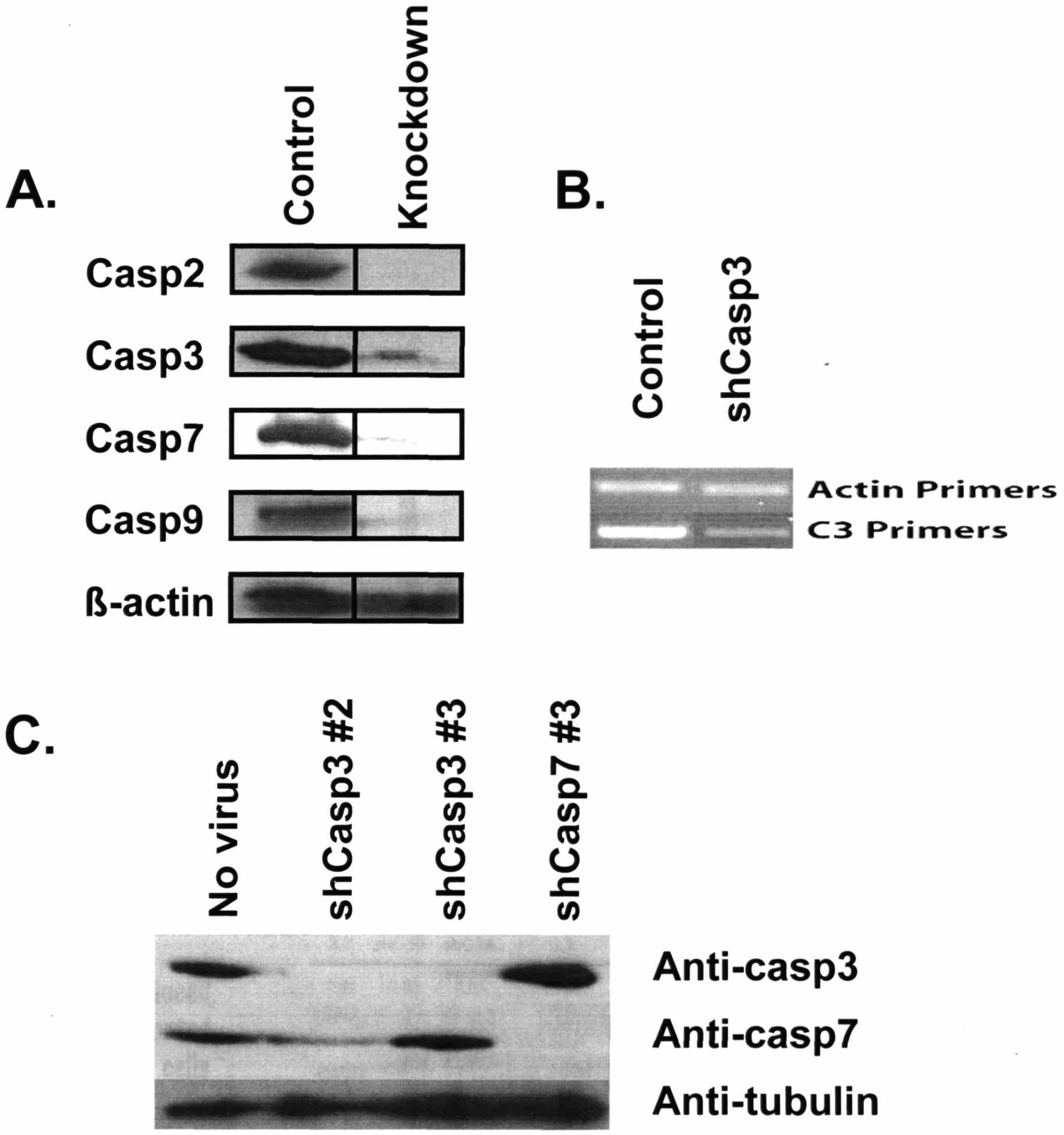


Figure 17. Validation of caspase shRNA sequences. shRNA sequences against murine caspases were designed using either Tuschl or Reynolds rules and cloned into pLL3.7. DO11.10 T cells were infected with pLL3.7 viruses containing putative caspase silencing shRNAs and were purified in a high speed cell sorter on the basis of GFP expression before Western or RT-PCR analysis. **A.** Silencing of caspase expression in the pLL3.7 infected knockdown DO11.10 cell lines by Western blot. **B.** RT-PCR analysis of caspase-3 expression in DO11.10 cells infected with pLL3.7 shCasp3. **C.** Validation of additional shRNA sequences targeted against caspase-3 and caspase-7 in pLL3.7 infected DO11.10 T cells lines by Western blot. Expression of both caspase-3 and –7 are evaluated in the same sample in this panel.

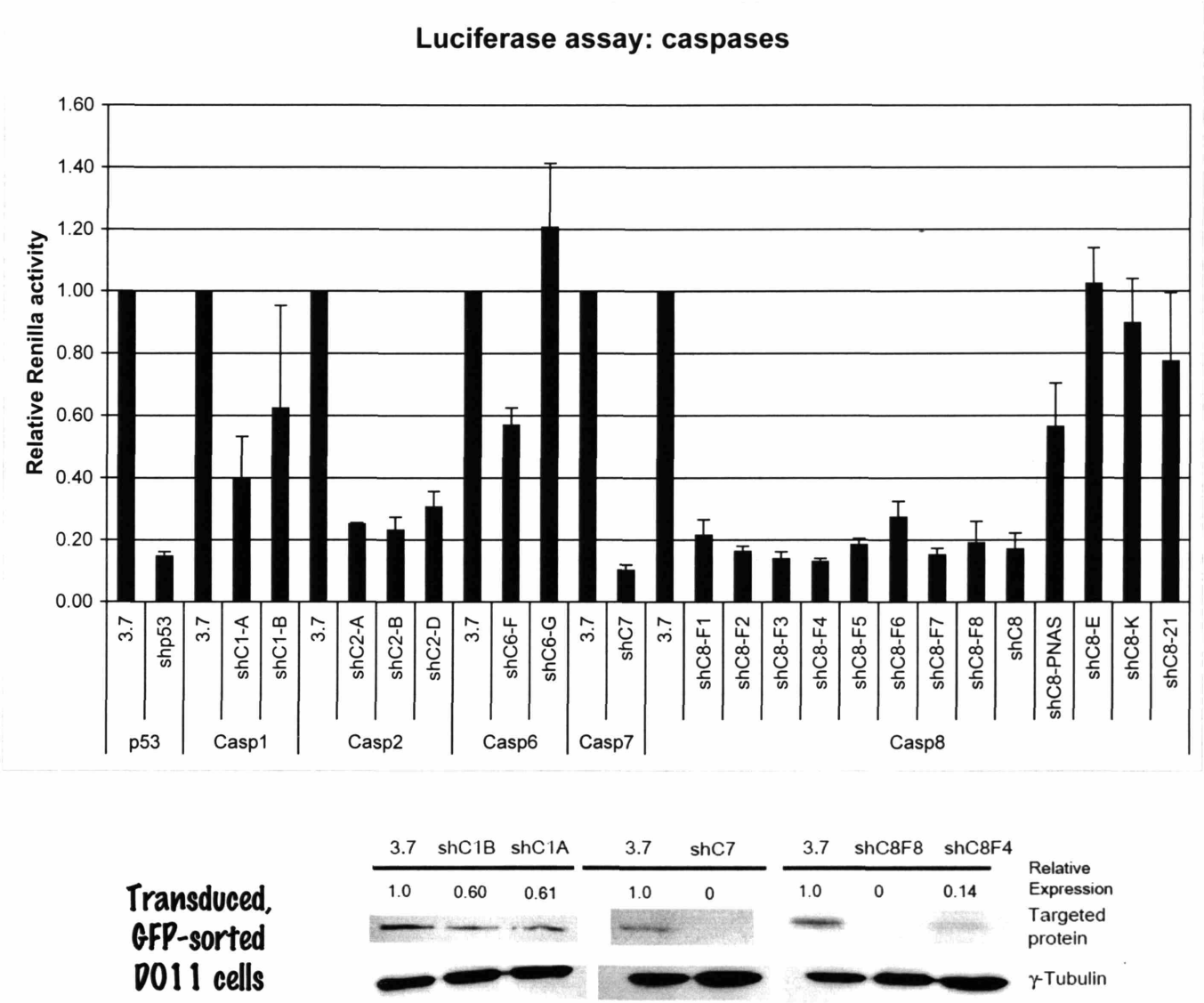


Figure 18. Validation of caspase silencing by pLL3.7 expressed shRNAs. **Top:** pLL3.7 vectors containing putatively silencing shRNA sequences are co-transfected with dual luciferase constructs containing the cDNAs of the targeted genes in the 3' UTR of Renilla luciferase into 293FT cells. The enzymatic activity of both luciferases are measured and normalized to the non-targeted firefly luciferase activity. Renilla luciferase activity is then normalized and compared to empty pLL3.7 vector to determine the effectiveness of silencing. The five caspase 8 knockdowns on the right were from the initial set of 7 that were tested. **Bottom:** DO11.10 cells were infected with selected construct, purified through high speed cell sorting, and analyzed via Western blot to confirm the luciferase results.

		Knockdown validated by (>50% reduction)		
Caspase	**# shRNAs**	**Western**	**RT-PCR**	**Luciferase**
1	2	0/2	-	1/2
2	3	2/3	-	3/3
3	3	3/3	1/1 (only 1 tested)	-
6	5	1/5	-	0/2
7	2	2/2	-	1/1 (only 1 tested)
8	15	2/9 (only 9 tested)	-	9/13 (only 13 tested)
9	5	1/5	-	-
11	2	-	-	-
12	2	-	-	-

Table 2. Summary of caspase shRNA validation results. Each caspase had the indicated number of shRNA sequences designed to target it. These shRNAs sequences were validated by infecting DO11.10 T cells and performing Western blots or RT-PCR on cell extracts. Alternatively, some of the sequences were tested via luciferase assay shown in Figure 18. Knockdowns were determined to be successful if silencing was greater than 50% in order to compare to results from Reynolds et al [33]. A full list of sequences tested appears in Appendix E.

After creating effective shRNA vectors against all caspases, I attempted to silence multiple caspases within the same cell line using two different approaches. The first approach was to produce shRNA transcripts from two separate U6 promoters located on the same vector. I cloned a second U6 promoter into pLentiLox 3.7 in the opposite orientation from the original U6 promoter with a short spacer sequence of 100 bp inserted between the promoters in order to reduce competition effects (Figure 19A). After cloning both shRNAs into the vector, I infected DO11.10 T cells and purified populations through high speed cell sorting. Western blot analysis performed on protein lystates from these cells showed that silencing was not effective for either of the two shRNAs expressed from this vector compared to controls that did not express a shRNA (Figure 20). It was not clear if either of the shRNAs were actually being expressed, but neither targeted protein was silenced.

Since the silencing was not effective using the dual U6 vector, we tried a second approach that used multiple vectors that each encoded a different reporter gene (Figure 19B). I generated virus for each gene and infected cells with two of the viruses simultaneously. Cells infected with both viruses were isolated via high speed cell sorting and protein lysates from these cells were used for Western blot analysis. Using this approach, the targeting of two shRNAs led to silencing levels similar to those observed when each shRNA was used individually (Figure 21). Thus, cells receiving the dual promoter vector were still capable of RNAi induced silencing from single U6 vectors, suggesting that the dual promoter vector itself was faulty.

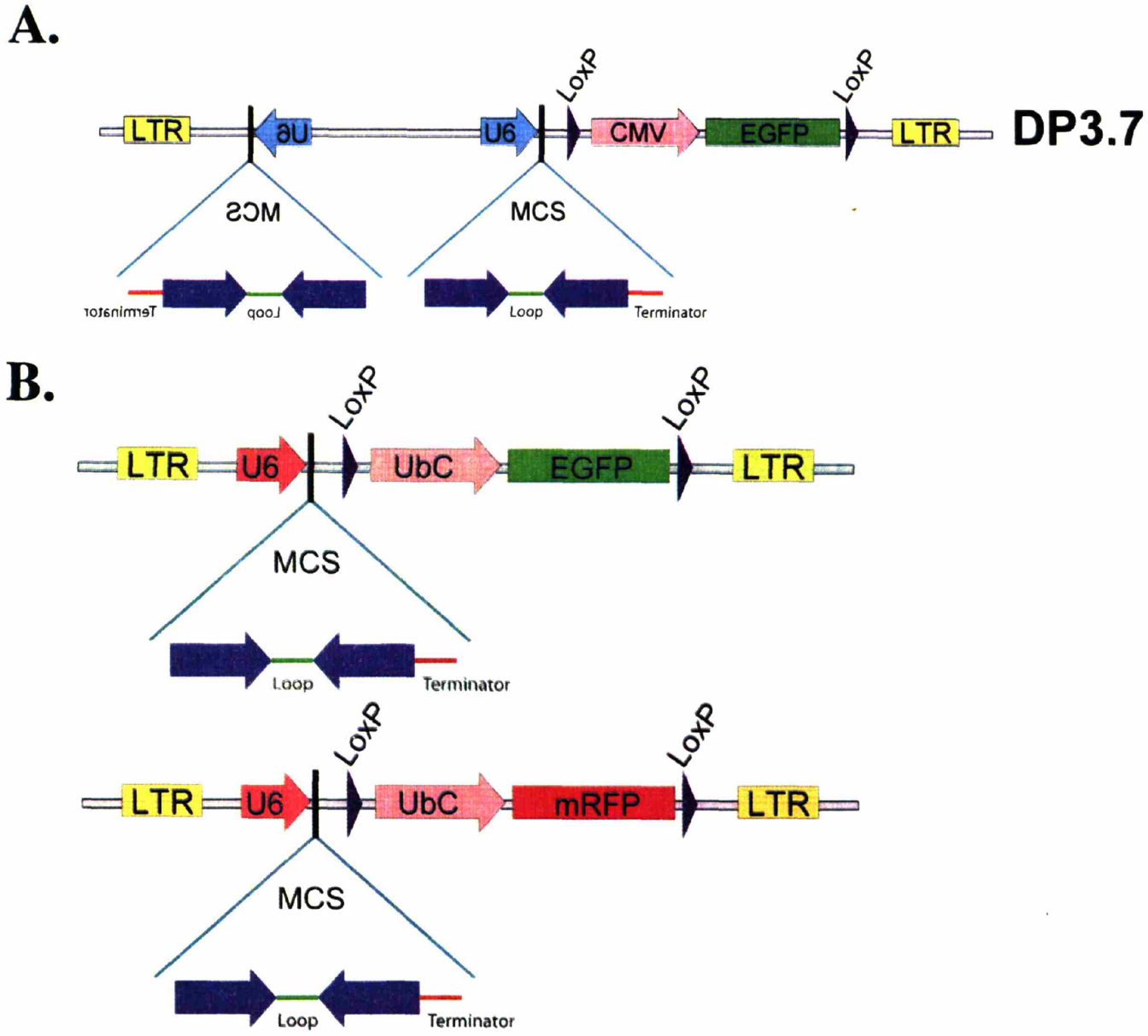


Figure 19. Schematics of vectors designed for silencing multiple caspases. **A.** The dual U6 LentiLox vector DP. A second U6 promoter is cloned in upstream and in opposite orientation of the original U6 promoter to enable expression of two individual shRNAs from the same viral integrant. **B.** Schematics of the pLBUG (top) and pLBUR (bottom) vectors. These vectors are variants of LentiLox 3.7 variants that use different fluorescent reporters (GFP and RFP respectively) for markers. These vectors also use the ubiquitin promoter in place of the CMV to drive reporter expression.

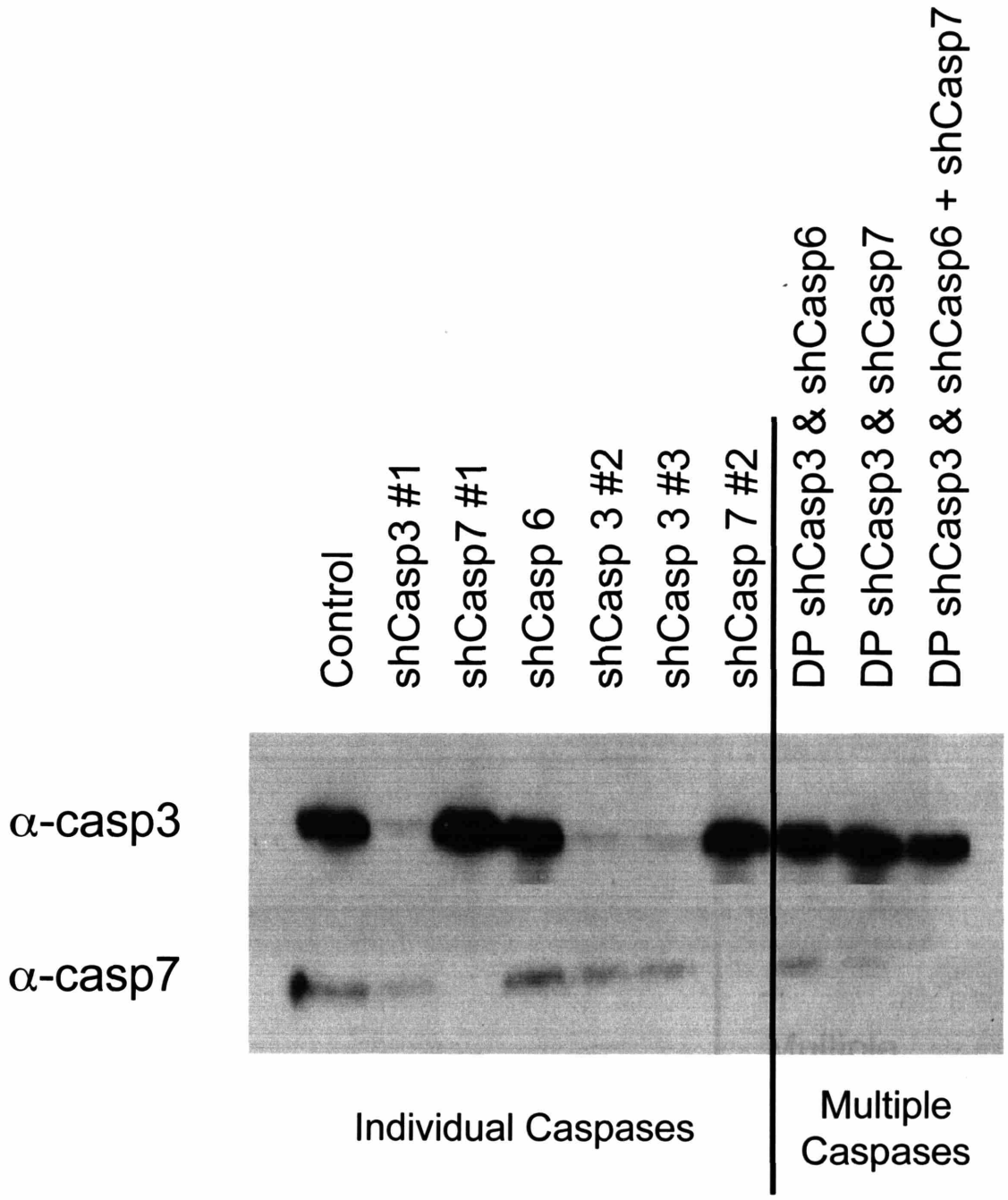


Figure 20. Silencing of effector caspase expression in the knockdown D011.10 lines. **Left:** DO11.10 T cells were infected with pLL3.7 viruses containing shRNAs against individual caspases. Western blots for caspases-3 and –7 were performed on cell extracts from cell lines purified on the basis of GFP expression by flow cytometry. **Right:** DO11.10 T cells were infected with LentiLox DP targeting caspases-3 and –6, caspases-3 and –7, or tandemly infected with DP targeting caspase-3 and –6 and pLBUR targeting caspase7 with the intention of generating double and triple knockdown cell lines. Cells were sorted to purify via flow cytometry on the basis of GFP and/or RFP expression and Western blots for caspase-3 and –7 were performed on cell extracts.

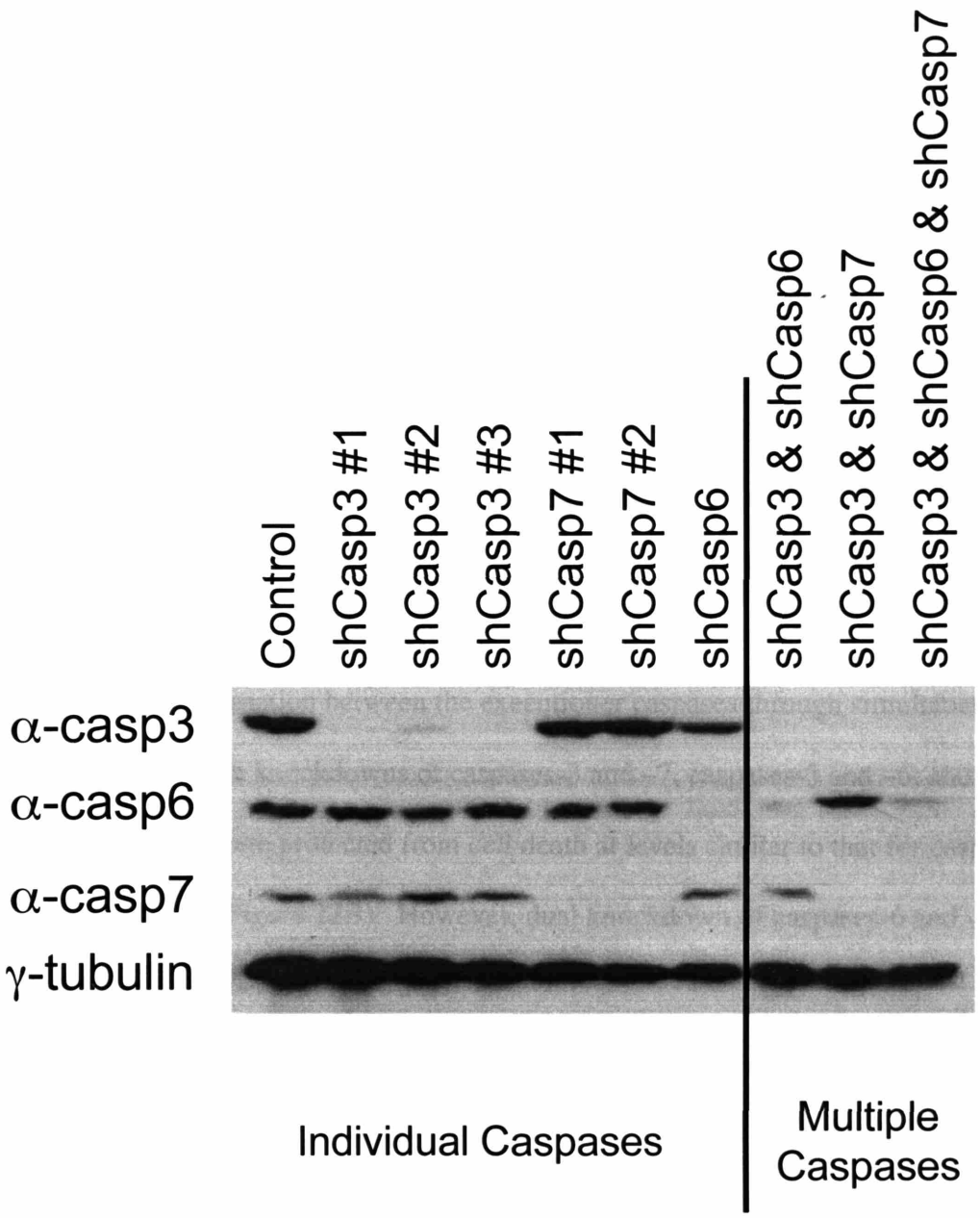


Figure 21. Silencing of effector caspase expression in the knockdown D011.10 lines. **Left:** DO11.10 T cells were infected with pLL3.7 viruses containing shRNAs against individual caspases. Western blots for caspases-3, -6 and –7 and gamma-tubulin were performed on cell extracts from cell lines purified on the basis of reporter expression by flow cytometry. **Right:** DO11.10 T cells were infected with combinations of pLBUG shCasp3, pLBUR shCasp7, and pLBUP (pLBUG with puromycin resistance replacing GFP as a marker) shCasp6 with the intention of generating double and triple knockdown cell lines. Cells were sorted to purify via flow cytometry on the basis of GFP and/or RFP expression and cells infected with pLBUP were treated with puromycin to select resistant clones. Western blots for caspase-3 and –7 were performed on cell extracts.

Apoptotic Assays

Purified DO11 cells infected with LentiLox vectors targeting individual caspases were treated with a series of death inducing stimuli, including anti-CD3, dexamethasone, etoposide, gamma irradiation, and 5-fluorouracil, in increasing concentrations or time periods. These cells were analyzed for apoptosis via flow cytometry using propidium iodide to exclude dead cells. Compared to controls, only caspase-3 knockdown cells showed significant protection ($p < 0.001$) against cell death when analyzed by two-way repeated ANOVA (Figure 22A). Knockdown of each of the other caspases individually did not appear to have any significant effect on cell death (Figure 22A). I investigated the potential for compensation between the executioner caspases through simultaneous knockdowns. Cells with knockdowns of caspases-3 and –7, caspases-3 and –6, and caspases-3,-6, and –7 were protected from cell death at levels similar to that for caspase-3 alone (with $p < 0.001$) (Figure 22B). However, dual knockdown of caspases-6 and –7 did not protect against cell death (Figure 22B).

In vivo analysis

Since knockout of caspase-7 is embryonic lethal, I decided to investigate its role in immune cell development using a lentiviral shRNA transfer vector to generate transgenic animals and chimeras deficient in caspase-7. After multiple viral injections, only 2 transgenic animals were created, both with low levels of GFP expression. One of these animals failed to breed, and the other produced a single transgenic offspring. This pup had the same profile of MFI as its parent, but approximately double the percentage of GFP positive cells (14%).

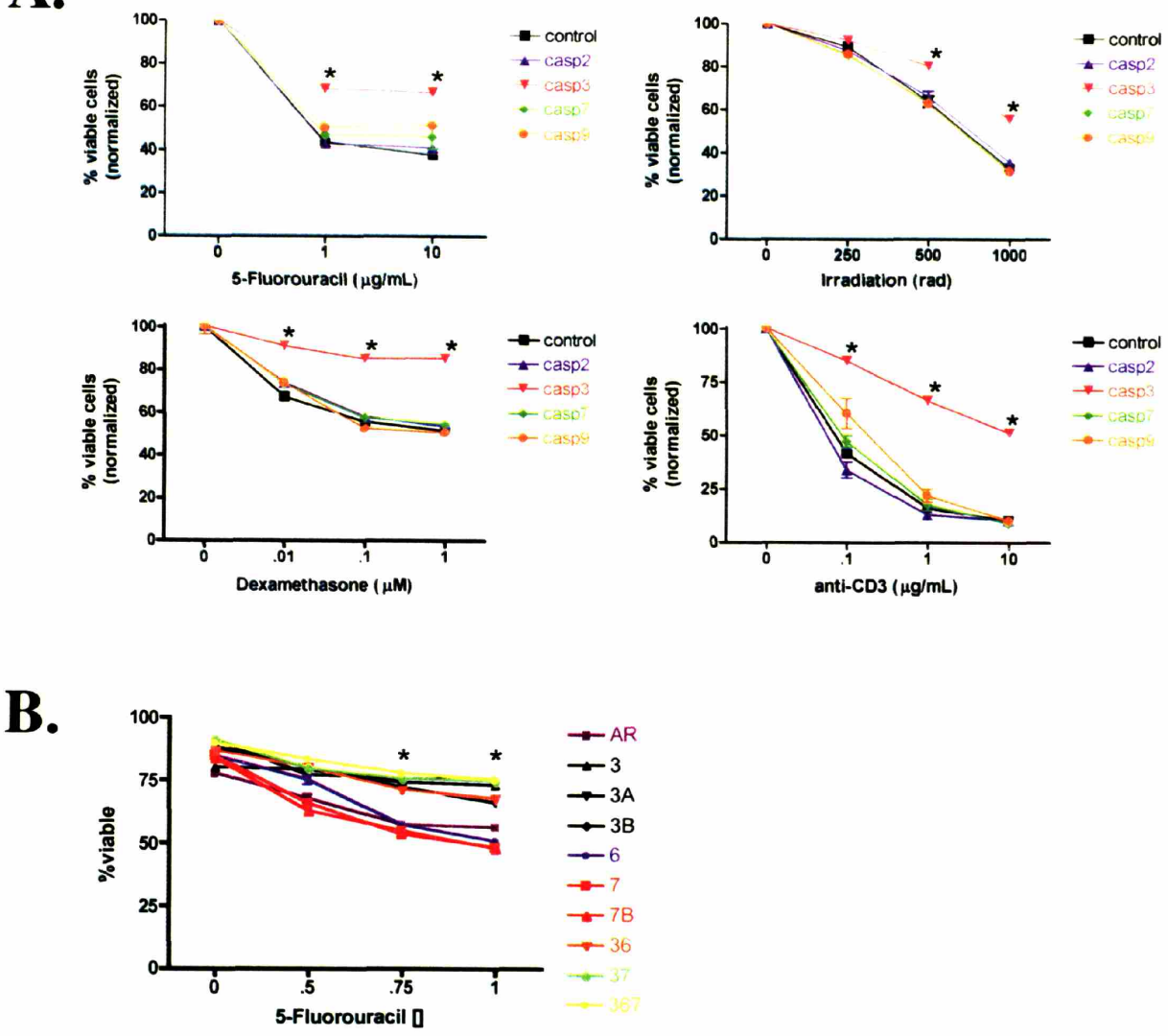


Figure 22. Protection against apoptosis in caspase knockdown lines. DO11.10 T cells were infected with pLL3.7 RNAi viruses targeting the indicated caspases and sorted to purity on the basis of GFP expression. Cells were stained with propidium iodide and analyzed via flow cytometry to determine cell viability 24 h (or 36h for gamma irradiation) after treatment. Each point on the curve represents triplicate samples and include error bars. Curves were compared using the two-way repeated measure ANOVA statistical test followed by Bonferroni post-tests to compare individual points on separated curves. Asterisks indicate that the curves are significantly different from control with a p value < 0.001. **A.** Silencing of caspase-3 protects against cell death induced by 5-fluorouracil, gamma irradiation, dexamethasone, and CD3 ligation. **B.** Silencing of caspase-6 or caspase-7 in caspase-3 knockdown cells does not provide additional protection against 5-fluoruracil induced cell death. However, the asterisks denote that all caspase-3 silencing vectors are significantly protected compared to control cells at a p value of <0.001.

As the transgenic approach proved more difficult than anticipated, I decided to generate bone marrow chimeras to produce animals in which a larger number of cells expressed the reporter and shRNA. I infected hematopoetic stem cell enriched bone marrow with either control virus or virus containing shRNAs against caspase-7. The infected cells were injected into irradiated recipients, which were screened for efficient bone marrow engraftment. The chimeras were then euthanized and their organs were analyzed via flow cytometry.

Analysis of the caspase-7 knockdown bone marrow chimeras showed that B cells and single positive T cells existed in both the blood and the peripheral lymph organs. Western blot analysis of both B and T cells purified from these animals showed an approximately 75% reduction caspase-7 expression (Figure 23). To determine if there was any perturbation in T cell development, thymocytes from four caspase-7 knockdown and four control animals were stained with appropriate markers and analyzed via flow cytometry (Figure 24A). The percentage of each thymic cell subset (DN, DP, CD4SP, and CD8SP) in knockdowns animals was not altered compared to controls (Figure 24B). In the spleen, however, there was an apparent increase in the percentage of B cells in knockdown animals compared to controls (Figure 25). To further characterize the observed increase in B cell percentage, I looked for variations in the size of the B cell subset populations in the bone marrow and spleen of these animals. While no difference was observed in the bone marrow B cells, there was a higher percentage of the IgM^- $B220^+$ B cell population in the spleens of transgenic caspase-7 animals compared to controls (Figure 26).

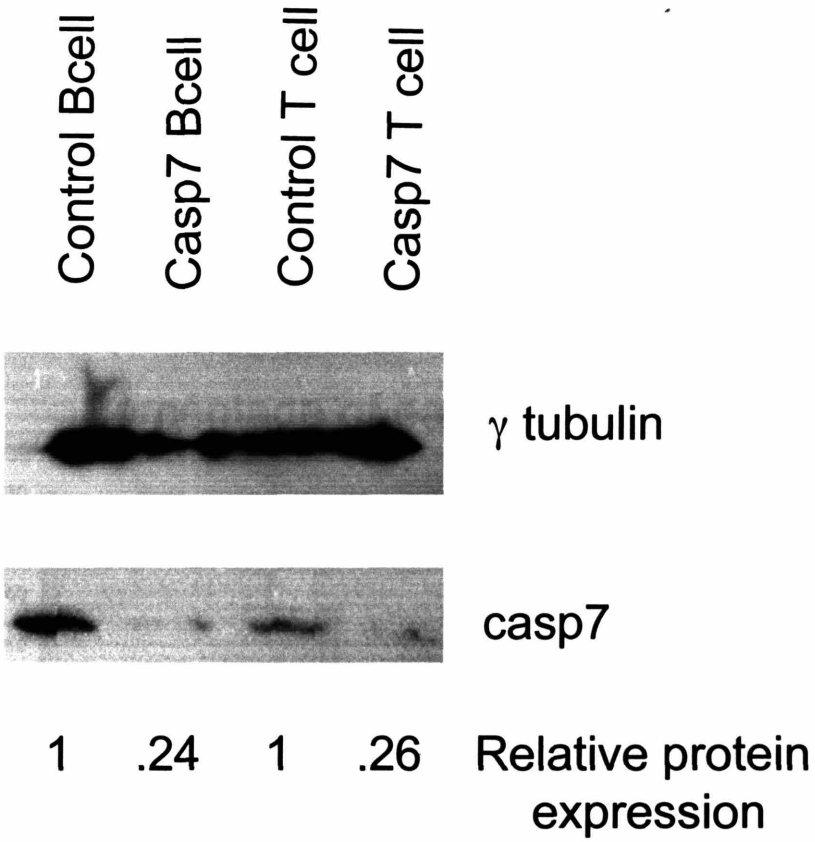
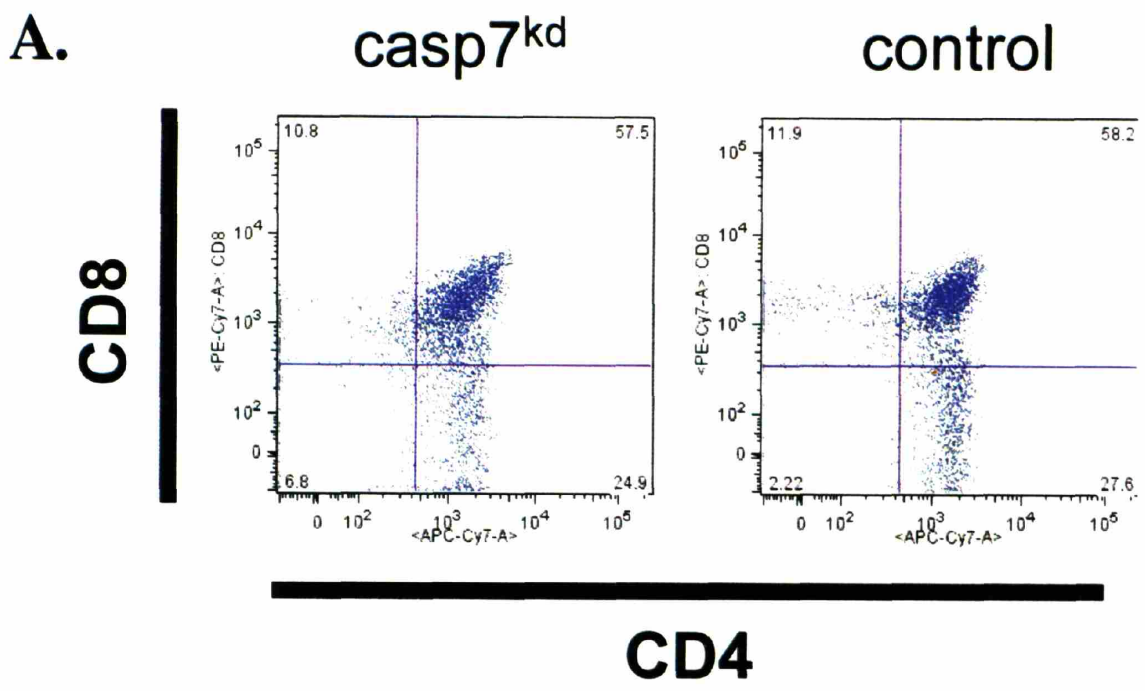


Figure 23. Silencing of caspase-7 in mature T and B cells isolated from knockdown and control bone marrow chimeras. Bone marrow was isolated from DO11.10 T donors, infected with pLL3.7 or pLL3.7 shCasp7, and injected into lethally irradiated Balb/c recipients. Western blots for caspase-7 expression were performed on extracts from T and B cells purified by flow cytometry from the spleens of 3 reconstituted animals.



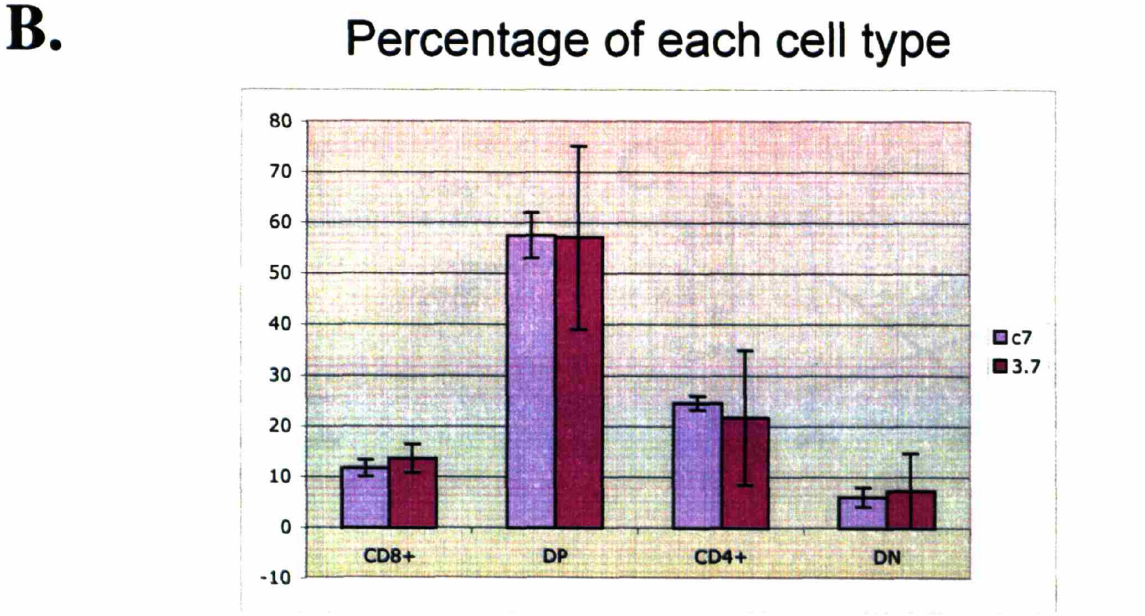
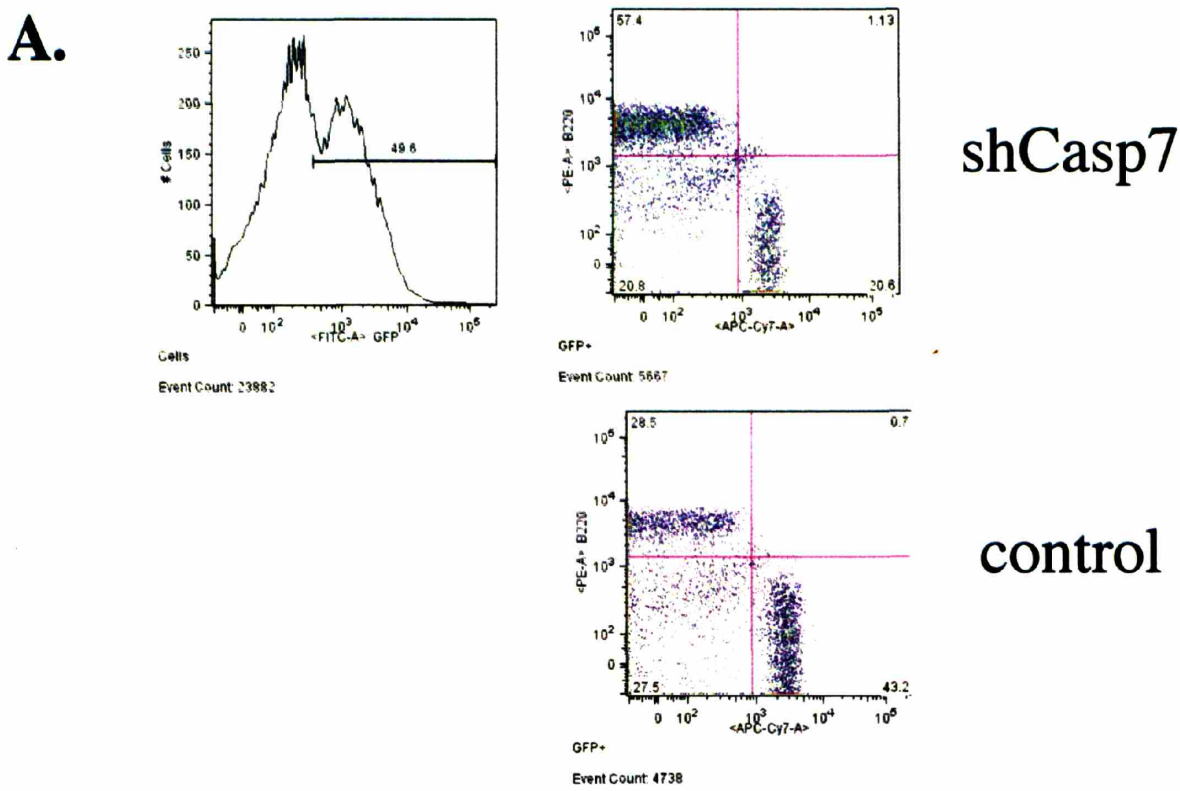


Figure 24. Caspase-7 does not affect the development of T cells. **A.** Expression of CD4 and CD8 in thymocytes from control and caspase-7 knockdown chimeras. Bone marrow was isolated from DO11.10 T donors, infected with either pLL3.7 or pLL3.7 shCasp7, and injected into lethally irradiated Balb/c recipients. Thymocytes from these animals were stained with antibodies against CD4 and CD8 and analyzed via flow cytometry. Plots are representative of 4 control and 4 caspase-7 knockdown animals. **B.** Percentage of T cells subsets in caspase-7 knockdown and control chimeras. Graphs represents averaged expression from 4 control and 4 knockdown animals. DP = double positive = $CD4^+CD8^+$; DN = double negative = $CD4^-CD8^-$.



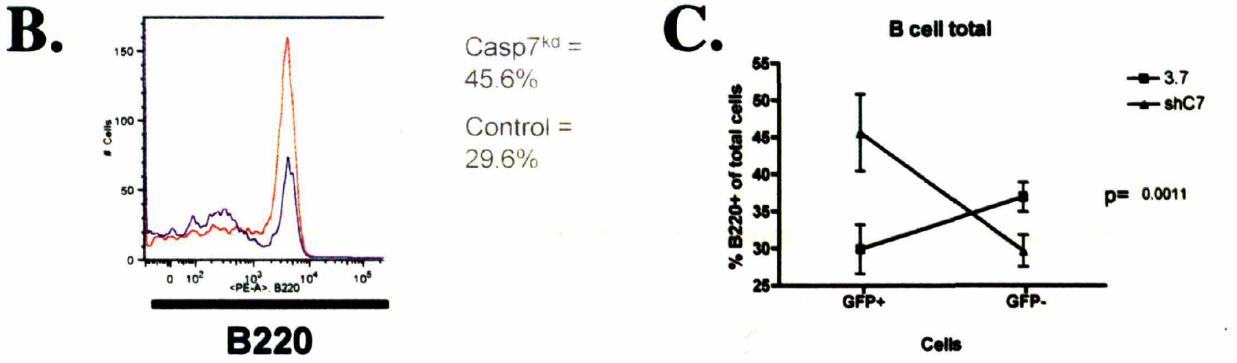


Figure 25. Caspase-7 ablation leads to expansion of the B cell compartment. **A.** Histogram of GFP expression in control and caspase-7 knockdown bone marrow chimera. Bone marrow was isolated from DO11.10 T donors, infected with either pLL3.7 or pLL3.7 shCasp7, and injected into lethally radiated Balb/c recipients. Density plots on right show B220 and CD4 expression for pLL3.7 shCasp7 (top) and pLL3.7 control (bottom). Plots are representative of 4 individual knockdown and control chimeras **B.** Histogram of increased percentage of B cells in representative caspase-7 knockdown (red line) chimera compared to control (blue line) animals from part A. **C.** B cells expansion in the GFP expressing population of caspase-7 knockdown chimeras compared to control chimeras. Two-way ANOVA analysis comparing effect of GFP and caspase-7 knockdown on the percentage of B cells in chimeric animals. Comparisons include 4 knockdown and 4 control animals.

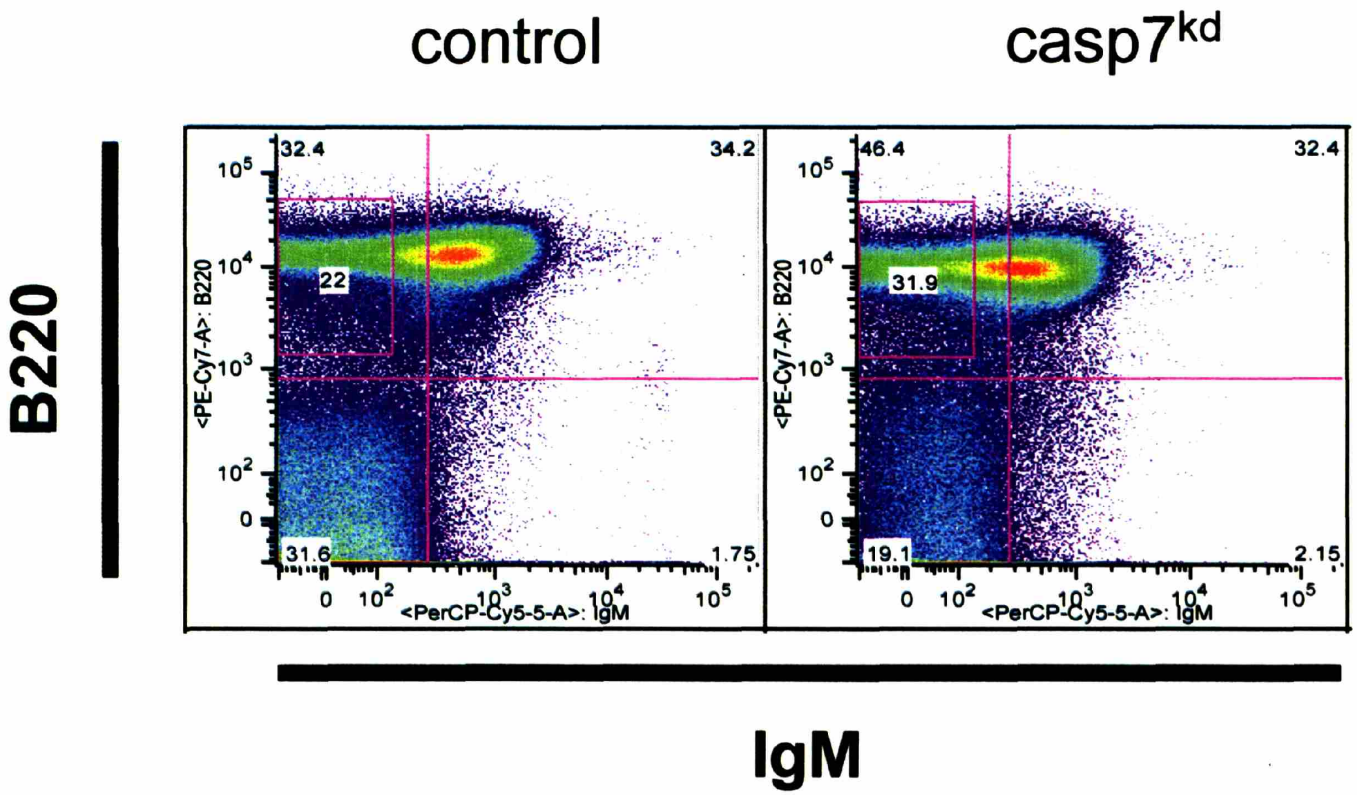


Figure 26. Expansion of IgM$^-$B220$^+$ splenocytes population in caspase-7 knockdown transgenics compared to control. Splenocytes from caspase-7 knockdown transgenics were harvested and stained with IgM and B220 antibodies and analyzed via flow cytometry. Plots are gated on GFP$^+$ cells only and are representative of two separate animals. Boxes in the upper left quadrant show IgM$^-$B220$^+$ cells.

Discussion

Despite the small size of the caspase family, its members have similar functions and thus overlap or compensation can obscure analysis of the functional loss of any particular caspase. To clarify the analysis of caspase function, I created a set of RNAi reagents to target each of the nine murine caspases, which I used combinatorially to examine potential functional overlap. However, not all the caspases proved to be amenable to knockdown by RNAi. Caspase-8 and its homologue FLIP, proved resistant to targeting as none of the sequences initially tested for either gene silenced its target. Eventually, we used a different design algorithm to find effective caspase-8 shRNAs sequences, which were validated by a colleague in the lab using a luciferase reporter assay. However, there is anecdotal evidence that some sequences are not targetable by RNAi, but no explanation for this phenomenon has been reported. My attempts to silence caspase-8 using two validated sequences from the literature failed [145, 146]. The first sequence was from a siRNA that I adapted into a shRNA for use in pLL3.7, which may have disrupted its efficacy. It is also possible that the DO11.10 cell line used for validation had polymorphisms from the consensus genomic sequence, as the second sequence was successfully used as a shRNA in another murine cell line.

Using a number of different stimuli to induce apoptosis in DO11.10 cells, only knockdown of caspase 3 rescued cells from cell death. Caspase-9 was expected to protect against cell death initiated through the mitochondrial pathway induced by gamma irradiation. There are several potential explanations for this observation. In DO11.10 cells, other caspases might not be involved in any form of apoptosis that was induced. Perhaps using other cell types or death inducers might elucidate the role of other

caspases. Alternatively, the level of knockdown of these caspases might not have been sufficient to protect against cell death, as there may have been enough protein spared to initiate the caspase cascade. This could explain why caspase-9 knockdown did not block cell death initiated through the mitochondrial pathway induced by gamma irradiation. Another possibility is that the signal for death induction was so strong that the cells apoptosed despite the mild protective effects of a caspase. Since the caspase activation cascade is an enzymatic process capable of rapidly activating many substrates, only a small amount of caspase activation may be required to induce cell death. A partial caspase-9 knockdown could be enough to protect against a milder inducer of cell death but a strong dose of radiation may have activated the little caspase-9 that remained, leading to cell death. However, this explanation seems unlikely as no protective effect was seen at the lower levels of the dose response curve for any of the death inducing stimuli.

For a few of the caspases, such as caspase-3 and –7, I generated multiple shRNAs that silenced the genes very effectively. Since cells silenced for the same gene with different shRNAs reacted similarity in response to death inducing agents, it is likely that off-target silencing effects are not playing a significant role in the apoptotic phenotype in these cells. Based on the Western blot analysis for caspase-3 and –7, the targeting of a single caspase did not appear to affect the expression level of the other caspases, showing that at least for the caspases of interest, there were no off-target effects. Thus, it appears the ablation of a single caspase does not cause compensation by increasing the protein expression levels of other caspases.

In attempts to knockdown two caspases simultaneously, the dual U6 promoter vector failed to silence the genes it was targeting. This effect was vector specific, not a widespread inhibition of RNAi since two vectors infected separately and targeting different genes silenced both targets effectively within a single cell. Additionally, cells were purified on the basis of reporter expression from the pol II promoter, showing that the virus was competent for infection and expression. This suggests that the design of the dual U6 vector was faulty. This could be a result placing the second promoter in the opposite orientation to the first or not leaving enough space between the promoters to prevent competition. While these experiments demonstrate that dual knockdowns are possible using two vectors, further experiments are needed to determine if silencing is as efficient with two vectors as when each gene is silenced separately.

I decided to examine executioner caspases because their sequence similarity might be a sign of functional overlap, especially between caspases-3 and –7. When I combined the caspase-3 knockdown with the silencing of either caspase-6 or -7, I found no increased protection against apoptosis, suggesting that the cells were either already maximally protected against apoptosis, or that the second caspase was not involved in the induced pathways. Since the knockout is embryonic lethal, caspase-7 must have an important role *in vivo*. To investigate this function, I generated transgenic animals by injecting virus into fertilized embryos. However, the mice generated from these injections had low GFP expression levels. I believe that performing additional embryo injections would have yielded progeny whose reporter expression was high enough for analysis. I generated six caspase-7 knockdown and six control bone marrow chimeras. However, expression levels were still lackluster (under 50% GFP+). Use of the same

protocol with other retroviral constructs led to the production of high expressing mice in previous experiments. In addition, production of lentiviral chimeras using the CD4-GFP vector were successful, suggesting there might be specific problems with the lentiviral backbone used for these experiments. Further testing of the lentiviral variants should answer these questions.

Despite the low expression levels, I characterized the phenotype of the chimeric animals. Caspase-7 did not seem to be required for the development of either T or B cells. Both T and B cells showed about 75% reduction in caspase-7 expression, so if caspase-7 was required for the development of these cells, I should not have found mature cells of both types in the knockdown animals. It is possible that caspase-7 is required for development, but enough caspase-7 remained to allow development to proceed normally. A more effective shRNA or a complete knockout would answer this question. No effect was observed on T cell development in the thymus, which was expected given the lack of effect of caspase-7 on apoptosis in the T cell line. Early B cell development was also unaffected, as there was a slight increase in the B cell compartment in the spleen of the chimeric animals, but no major changes in the bone marrow. This increase in B cells appeared to be due to an expansion of the nearly mature $IgM^{-}B220^{+}$ B cells population, suggesting that loss of caspase-7 enables B cells undergoing negative selection to preferentially survive. This explanation is consistent with data from the WEHI-231 line, whose immature B cell phenotype is used as a model of B cell tolerance. In this model, caspase-7 is preferentially activated after engagement of the B cell receptor by anti-IgM antibodies [141]. An alternative explanation for the increase in B cell percentage would be a decrease in percentage of T cells. These two

explanations could be differentiated by counting the number of T and B cells in each animal rather than analyzing the percentage of each cell type. The failure to do this in these experiments was an oversight that can be corrected when analyzing animals in the future. Further examination of this model along with *ex vivo* or cell line apoptosis assays is needed to illuminate the complete mechanism behind this phenotype.

The expansion of the B cell population might result from the protection that caspase-7 abaltion offers when the cells are stimulated for cell death. Another possibility is that caspase-7 causes defects in another cell type, such as dendritic cells or T cells, that allow more B cells to survive, perhaps by not inducing cell death when they should or by an increase of cytokine signals when they should not provide them. This second scenario, known as cell-extrinsic effects, can only be separated from the first scenario (cell intrisinic effects) by tissue specific deletion of caspase-7. If caspase-7 was eliminated only in B cells through the use of a B cell specific Cre with floxed caspase-7 and there was still an increased proportion of B cells, then it could be concluded that the process was cell-intrinsic. Otherwise, tissue specific ablations of caspase-7 in other cell types would be required to determine how this caspase mechanistically affects the phenotype.

Clearly, further experiments are necessary to determine how closely RNAi knockdown animals phenocopy their respective knockouts. Since caspase-3 knockouts have been well characterized, comparison of these animals to caspase-3 knockdowns should provide preliminary insights. If large differences exist between the phenotypes, it should give pause to the exclusive use of RNAi knockdown animals to diagnose gene function.

Chapter 5

Discussion

RNAi is a rapidly advancing field, where many key insights are still being elucidated. The field is currently bifurcated between those studying the mechanism of RNAi and those using RNAi as a research tool. Insights into RNAi mechanisms have and will continue to inform the use of RNAi as an investigative tool, resulting in the design of increasingly specific and effective siRNAs and shRNAs. Improved delivery methods for the efficient transduction of RNAi inducing nucleic acids, such as viral vectors, will further increase the utility of RNAi techniques. In this thesis, I have demonstrated that lentiviruses are effective delivery tools for RNAi, increasing the pace at which transgenics can be created.

Using the lentiviral shRNA system I developed, I began to investigate the role of caspases in immune function. My work showed that the only caspase knockdown that protected cells from apoptosis induced by a variety of death inducers was caspase-3. No additional protective effects were seen when caspase-6 or caspase-7 was simultaneously knocked down with caspase-3. I would be interested in extending these experiments using other cell types, particularly B cells or B cell lines since there is evidence that caspase-7 might play an important role in apoptosis in these cells. Given time restraints and difficulty with mouse production, my *in vivo* analysis of caspase-7 was not as complete as I would have liked. Ideally, I would use the improved lentiviral vector pLB to produce transgenic caspase-7 knockdown animals, since this would be the best way to examine how caspase-7 functions in B cells. While I have preliminary evidence suggesting that caspase-7 protects immature B cells from elimination, flow cytometric

data and cell counts from a larger cohort of animals are needed to make any firm conclusions. I would also like to perform *ex vivo* apoptosis experiments on caspase-7 B cells to see if they are resistant to cell death induced by ligation of the B-cell receptor, the likely signal for B-cell selection. If expansion of the B-cell subset was confirmed, I would then try to determine whether this was a cell intrinsic or extrinsic effect. To do so, I would use the pSICO vector to generate chimeras by infecting bone marrow from CD19-Cre animals or generate a pSICO shCasp7 transgenic and breeding it with the CD-19-Cre animals. If the expansion of B cells was seen in these animals, this would confirm that the phenotype was cell intrinsic. I would then cross the transgenic knockdown animals into a B-cell receptor transgenic system, such as the hen egg lyzozyme (HEL) specific B-cell receptor transgenic, to determine if antigen-specific B cells would escape selection. If the B-cell effect disappeared in the CD-19-Cre animals, I would cross transgenic pSICO animals to other strains expressing Cre in specific hematopoetic compartments to isolate which cell population extrinsically controlled the expansion of B cells. These experiments would give strong functional evidence that caspase-7 is important in immune development.

While investigations of immune system function can benefit from the use of RNAi technology, scientists must consider the current limitations of these techniques. The efficacy of RNAi, especially from tissue to tissue, is variable and not well characterized. Within an animal, an shRNA that silences effectively in immune cells might not silence to the same degree in lung or muscle tissue [147]. Variations in silencing could be a result of differential processing of the shRNAs from tissue to tissue. Strict characterization of the cleavage products from shRNA stem loops in individual

tissues could inform the design of more efficient strategies for shRNA delivery. For instance, current efforts to develop systems in which shRNAs are delivered in a miRNA-like context from pol II promoters will allow shRNAs to be processed in a natural cellular context and should improve silencing efficiency.

In addition to efficacy, an shRNA must also specifically silence its target to be useful for scientific experiments. Specificity of shRNAs is especially important when characterizing genes of unknown function whose ablation might lead to unexpected phenotypes since it is important to distinguish between relevant phenotypes and those caused by off-target effects. *In vitro*, the use of multiple targeting sequences for the same gene can demonstrate that observed phenotypes are attributable to silencing of the targeted gene rather than the use of a particular shRNA. However, it remains unclear if all shRNAs validated *in vitro* will silence with similar efficacy *in vivo*. While the hurdles of using this approach *in vivo* are significant, comparing RNAi animals targeting different regions of the gene of interest would be the most convincing demonstration of a phenotype's veracity.

Lentiviruses pose their own challenges when used as a delivery mechanism for transgenesis. Even though it has been three years since the creation of the initial lentiviral transgenic by Lois et al[49], the stability of expression through breeding is still being investigated. The variability of expression between generations, particularly the rise of reporter expression from the founder to F1 progeny, is not understood. This might result from stochastic gene silencing mechanisms that are reset during embryogenesis in progeny generations enabling higher reporter expression. In addition, it is unclear how many integrants are required for substantial reporter expression or if interactions between

separate integrants might influence expression. As a result of this variation, lentiviral expression in transgenics and chimeras never reached 100%, so the effectiveness of this technique for studying non-cell autonomous processes is limited. Biosafety concerns and titer requirements for the use of lentiviruses might also limit their general applicability for biological investigations.

Is transgenesis by pronuclear injection an obsolete technique, surpassed by the new technology of lentiviral transgenesis? Given my experience with lentiviral transgenesis, I would argue that proclaiming the death of pronuclear injection would be premature. Despite these limitations, lentiviral transgenesis has several advantages compared to pronuclear injection. While lentiviruses do not integrate into the genome as concatamers like DNA from pronuclear injection, multiple viral integrants often need to be separated through breeding. Lentiviruses also enable the creation of transgenics in diverse species, like rats, and strains, especially those with poorly distinct pronuclei, that are impervious to pronuclear injection. Furthermore, the same lentiviral construct can be used for embryo injection, infection of ES cells, or experiments *ex vivo* in primary cells, whereas constructs for pronuclear injection do not have the same flexibility.

While lentiviruses are useful for many investigations, pronuclear injection is advantageous for some experimental models. The construct for pronuclear injection can be much larger, typically up to 20 kb but as large as 200kb [148], and can include more regulatory elements, multiple promoters, and even introns, which would interfere with lentiviral packaging. Animals generated from pronuclear injections are thought to have more consistent transgene expression across tissues compared to lentiviral transgenics, however, few studies have formally quantified expression throughout animals derived by

either method. Our data shows that even using vectors with anti-repressing elements does not result in every cell expressing from the viral integrant. The pronuclear injections might be preferable to lentiviruses in experimental models where expression is needed in every single cell for the phenotype to occur. It is not generally recognized in the research community that expression from the lentivirus is as variegated as it is, and this will hinder the use of lentiviral transgenics for disease study.

The technical challenges and safety issues continue to make lentiviral work difficult. While both lentiviral infection and pronuclear injection require specialized equipment and a well-trained injectionist, virus must be harvested from transient transfections and highly concentrated to achieve titers necessary for embryo infections, whereas pronuclear injection only requires preparation of DNA. The concentration of lentiviruses requires VSV-G, whose tropism enables humans to be infected, and has caused ongoing debate about the biosafety of targeting by RNAi unknown human genes that might have tumor suppressor function. Separate production facilities are maintained to ensure that exposure to these viruses is limited. These technical and biosafety issues have slowed the adoption of lentiviruses for generating of transgenic animals. Given these caveats, I would recommend the use of pronuclear injection in strains that are easy to target or where multiple regulatory components are required in the transfer vector and the use of lentiviral transgenesis in difficult to manipulate animal models.

Chimeras from ES cells are also an alternative to lentiviral transgenesis. While lentiviruses can be used to generate knockdown animals, the targeting of genes in ES cells is the primary way in which knockouts are created. Lentiviruses can be used to infect ES cells for generating chimeras, however, the direct infection of fertilized

embryos is probably more efficient since the resultant offspring can be directly used as founders without the need to determine if the integrant has gone germline. Targeting constructs in ES cells directly eliminate important exons of a gene ensuring that gene expression is completely ablated in every tissue, while lentiviruses will only silence expression in cells where the viral integrant is expressed. Furthermore, using RNAi based silencing can complicate analysis in cases where the knockdown may leave enough residual activity for the gene to fulfill its function. Thus, ES chimeras are useful for generating knockouts to examine the role of one or two genes in normal strains. However, when ES cells do not exist, such as for the non-obese diabetic (NOD), lentiviral transgenesis provides a strong alternative method for generating knockdown animals. In addition, lentiviral transgenesis might be more useful than ES targeting for probing the function of a larger number of genes as when following up top hits from a screen.

RNAi is a promising new technique that could enable more efficient and effective gene analysis on highly specific backgrounds, such as complex disease strains, where the exact combination of genes would be hard and slow to achieve through traditional breeding. For example, the NOD diabetic mouse, where nineteen loci contribute to the onset of disease, is an ideal candidate for RNAi manipulation. In addition, as demonstrated in this thesis, RNAi can be used to simultaneously knockdown two or more genes. As gene families have enlarged and diversified from their primordial ancestors in worms and flies, the ability to individuate the roles of each member gene becomes more difficult. Multiple gene knockdowns can improve our understanding of the function of

mammalian gene families, where potential overlap in function has limited the interpretation of studies using traditional methods

The future potential of RNAi and lentiviral transgenesis remains unknown. Efforts are underway to generate large libraries of lentiviral RNAi vectors to target the entire mouse and human genomes. It is too early to know if large-scale *in vivo* screens using these libraries will be possible given the technical hurdles of lentiviral work. I believe the most fruitful research advances using RNAi *in vivo* will examine small families of targets in a single disease model.

Technological advances often occur when we strive to realize our dreams of what could be possible, however, we must not lose sight of the utility of what is possible. While the ultimate potential for RNAi technology remains in the realm of possibility, it has already demonstrated uses that will greatly improve our understanding of gene interactions and functions for many years to come.

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Appendix A

"Small interfering RNA-mediated gene silencing in T lymphocytes."

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Small Interfering RNA-Mediated Gene Silencing in T Lymphocytes[1]

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Introduction of small interfering RNAs (siRNAs) into a cell can cause a specific interference of gene expression known as RNA interference (RNAi). However, RNAi activity in lymphocytes and in normal primary mammalian cells has not been thoroughly demonstrated. In this report, we show that siRNAs complementary to CD4 and CD8α specifically reduce surface expression of these coreceptors and their respective mRNA in a thymoma cell line model. We show that RNAi activity is only caused by a subset of siRNAs complementary to the mRNA target and that ineffective siRNAs can compete with effective siRNAs. Using primary differentiated T lymphocytes, we provide the first evidence of siRNA-mediated RNAi gene silencing in normal nontransformed somatic mammalian lymphocytes. *The Journal of Immunology*, 2002, 169: 5754–5760.

Introduction of dsRNA into an organism can cause specific interference of gene expression (1). This phenomenon, known as RNA interference (RNAi),[3] results from a specific targeting of mRNA for degradation by an incompletely characterized cellular machinery present in plant, invertebrate, and mammalian cells (2, 3). The proteins mediating RNAi are part of an evolutionarily conserved cellular pathway that processes endogenous cellular RNAs to silence developmentally important genes (4, 5). In RNAi, the protein Dicer, an RNase III enzyme, is probably responsible for the processing of dsRNA into short interfering RNA (siRNA). Functional screens conducted in plants and worms have identified a number of other conserved genes participating in the RNAi pathway. These genes include a number of different helicases, a RNA-dependent RNA polymerase, an exonuclease, dsRNA-binding proteins, and novel genes of unknown function (for recent reviews, Refs. 6, 7, 8, 9, and 10).

Mammalian RNAi was first described in mouse embryos using long dsRNA (11, 12). Then, following the analysis of the structure of the intermediate in this process, small interfering RNAs (siRNAs) were used to silence genes in mammalian tissue culture (13, 14). Most of the RNAi pathway genes discovered in plant and worm screens are also present in mouse and human sequence databases, supporting evidence that a conserved RNAi pathway exists in mammals. One of the more notable exceptions is the RNA-dependent RNA polymerase gene, which has been shown to be involved in the amplification of the dsRNA in *Caenorhabditis elegans* (15, 16). This might imply that perpetuation of the RNAi response in mammals differs from that of lower organisms.

Recent reports have demonstrated gene silencing by siRNA in mammalian cells (17–22). However, despite these initial reports, many uncertainties remain concerning the mechanism, physiologic relevance, and ubiquity of RNAi in mammalian cells. Although studies in tumor cell lines have demonstrated siRNA-mediated RNAi, it remains a major question as to whether primary cells from fresh tissues can undergo the RNAi response. Furthermore, little is known about the efficiency and longevity of siRNA-mediated RNAi gene suppression. In this report, we provide fundamental insight into the siRNA-mediated RNAi mechanism using a thymoma-derived cell line model to demonstrate for the first time the occurrence of RNAi in primary T lymphocytes.

Materials and Methods

Cell culture

E10 is an immature double-positive thymocyte line derived from a TCR-α and p53 double-mutant mouse of a mixed 129/Sv × C57BL/6 background as described (23). These cells, which proliferated vigorously, were maintained at a maximal concentration of 2×10^6 cells/ml and were propagated in complete medium: DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. Cell culture of primary lymphocytes: cells from the spleen and lymph nodes of DO11.10 TCR-transgenic mice (a generous gift from Dr. C. London, University of California, Davis, CA) were activated for 3 days with 1 μg/ml OVA peptide (residues 323–339) in RPMI medium containing 10% FBS.

Transfection

For electroporations, 2.5 μmol dsRNA and/or 20 μg of pEGFP-N3 plasmid (Clontech Laboratories, Palo Alto, CA) were added to prechilled 0.4-cm electrode gap cuvettes (Bio-Rad, Hercules, CA). E10 cells (1.5×10^7) were resuspended to 3×10^7 cells/ml in cold serum-free RPMI, added to the cuvettes, mixed, and pulsed once at 300 mV, 975 μF with a Gene Pulser electroporator II (Bio-Rad). Cells were plated into 6-well culture plates containing 8 ml of complete medium and were incubated at 37°C in a humidified 5% CO_2 chamber. Cell viability immediately after electroporation was typically around 60%. For cationic lipid transfections, 2 μg of plasmid DNA and 100 nmol siRNAs were used per 10^6 cells, and transfection followed manufacturer's recommended protocol. Transfection of primary lymphocytes: activated DO11.10 T cells were electroporated as above, except that the cells were resuspended to 6×10^7 cells/ml in cold serum-free RPMI and the pulse voltage was 310 mV. After electroporation, the cells were put into four wells of a 24-well plate, each containing 1 ml

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[3] Abbreviations used in this paper: RNAi, RNA interference; siRNA, small interfering RNA; PI, propidium iodide; GFP, green fluorescent protein; ORF, open reading frame; UTR, untranslated region; stRNA, small temporal RNA.

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of RPMI supplemented with 1 ng/ml IL-2 (BioSource International, Camarillo, CA). siRNA oligos (Dharmacon, Lafayette, CO) used were as follows (sense strand is given): effective CD4 siRNA, CD4 no. 4, (sense) gagccauaaucucaucugadgdg, (anti-sense) ucagaugagauuaauggcucdtdt; effective CD8 siRNA, CD8 no. 4, (sense) gcuacaacuacuacaugacdtdt, (antisense) gucauguaguaguuguagcdtdt; ineffective siRNAs, CD8 no. 1, (sense) gaaaa uggacgccgaacuudgdg, (anti-sense), aaguucggcguccauuuucdtdt; CD8 no. 2, (sense) cgugggacgagaagcugaadtdt, (antisense) uucagcuucucgucccacgdtdt; CD8 no. 3 (sense) aauuguguaaaauggcaccgcdcda, (antisense) μggcggugc cauuuuacacaadtdt; CD4 no. 1, (sense) ggagaccaccaugugccgadgdc, (antisense) ucggcacaugguggucucccdtdt; CD4 no. 2, (sense) ggcagagaaggauucu uucdtdt, (anti-sense) gaaagaauccuucucugccdtdt; CD4 no. 3, (sense) ccaccugcguccugucucadtdc, (antisense) gugguggacgcaggacagadgdt; CD4 no. 5 (sense) ccaccugcguccugucucadtdc, (antisense) ucagaugagauuaauggcucdtdt.

Flow cytometry

E10 cells (~1 × 10^6) were washed once in FACS buffer (PBS supplemented with 2% FCS and 0.01% sodium azide), resuspended to 100 μl, and stained directly with PE-conjugated anti-CD4 (clone RM4-5) or allophycocyanin-conjugated anti-CD8α mAbs, and in some experiments with PE- or allophycocyanin-conjugated anti-mouse Thy-1.2 (clone 53-2.1) mAb. All mAbs were from BD PharMingen (San Diego, CA). The stained cells were washed once, then resuspended in 200 μl FACS buffer containing 200 ng/ml propidium iodide (PI). Unstained and singly stained controls were included in every experiment. 3A9, a T cell hybridoma line that had been infected with a MIGW green fluorescent protein (GFP) retrovirus was included when GFP expression was analyzed. Cell data were collected on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and four-color analyses (GFP, PE, PI, and allophycocyanin) were done with CellQuest software (BD Biosciences). All data were collected by analyses performed on 1 × 10^4 PI-negative events (viable cells). For the primary T cell studies, activated cells were analyzed as above, except that allophycocyanin-conjugated anti-CD4 and PE-conjugated anti-CD8α were used, and 5 × 10^4 PI-negative events were analyzed.

Northern blot analysis of mRNA

Cells were lysed in TRIzol reagent (Life Technologies, Grand Island, NY) and total cellular RNA was purified according to manufacturer's instructions. RNA (10 μg) was fractionated on a denaturing 1% formaldehyde/agarose gel and transferred to a nitrocellulose membrane. Blots were hybridized overnight with ^{32}P-labeled CD4 (818 bp) or CD8α (596 bp) cDNA fragments. After washes, blots were analyzed by a PhosphorImager (Molecular Dynamics).

Results

siRNAs transiently induce silencing in murine thymocyte cell lines

To study RNAi, siRNAs are typically delivered into cells by carrier-mediated transfection reagents. We developed an experimental system using a thymoma-derived cell line, E10 (23), wherein we studied the use of siRNAs to silence either CD4 or CD8α, using the other marker as an internal specificity control. However, typical of lymphocytes, E10 is insensitive to several different cationic and noncationic transfection reagents and thus electroporation was used to introduce siRNAs. Using this method, ~20% of the cell population expressed GFP from a transfected reporter vector. When CD4 or CD8α siRNAs were electroporated into E10, a marked reduction in surface CD4 or CD8α expression, respectively, occurred 36 h later. Flow cytometry analysis showed that most of the cells were transfected and expression levels were reduced >5-fold below wild-type expression levels (Fig. 1*A*). The degree of reduction of CD8α was frequently more pronounced than that of CD4 and, in both cases, a small population of cells appeared to be either untransfected or not responsive to the siRNA treatment. In repeated experiments, typically 70–95% of the cells exhibited a >5-fold reduction in CD8α expression, although sometimes a smaller fraction of cells down-regulated CD8α to a greater degree (Fig. 1*A*).

Elbashir et al. (13) reported that RNAi-induced silencing could be maintained for ~2 wk in HeLa cells, although neither the extent of silencing nor the number of cell divisions was reported. A time

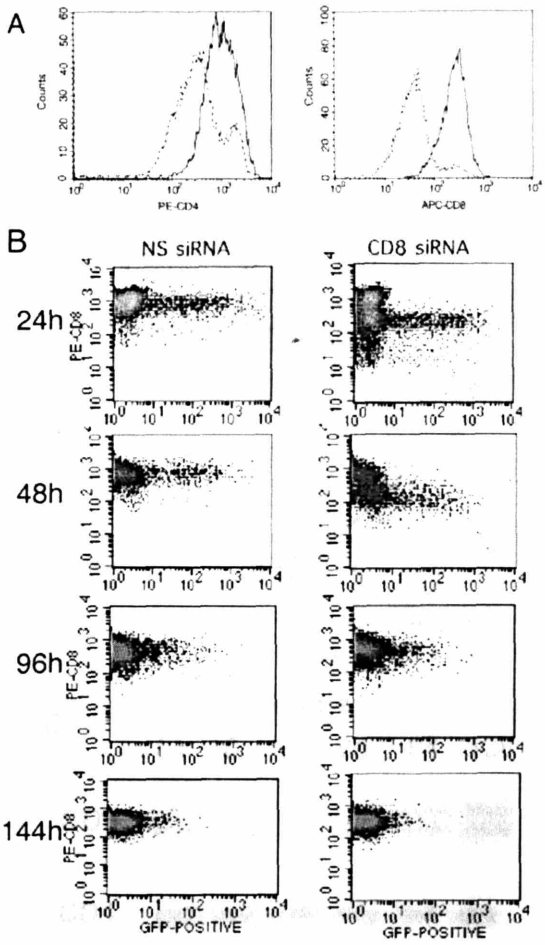

FIGURE 1. CD4 and CD8α siRNAs transiently silence gene expression. *A*, Histogram showing the typical reduction of CD4 and CD8α gene expression seen 36 h post siRNA transfection in E10 T cells. Solid and dashed lines indicate untransfected and siRNA-transfected E10 cells. Approximately 85% of $CD4^+$ cells and 94% of $CD8^+$ cells down-regulated surface expression. *B*, Flow cytometry analysis of transiently transfected GFP and either CD8α or luciferase siRNAs (NS siRNA) in the E10 T cell line. At 24 h, ~60% of the CD8 siRNA-transfected cells down-regulated surface CD8 expression. Gated live cells are shown, and the numbers at the left correspond to the times in hours after electroporation transfection.

course assay was performed in CD8α siRNA-transfected E10 cells. GFP was included in these transfections to investigate the relationship between the uptake and expression of plasmid DNA and siRNAs. Because these experiments were transient transfections, cell doubling results in a decrease in GFP fluorescence intensity and number of GFP-positive cells (Fig. 1*B*, NS RNA). When CD8α siRNAs were cotransfected with the GFP reporter vector, CD8α expression, but not GFP expression, was markedly reduced (see Fig. 1*B*, 24 h). Several cell populations were evident, with the major CD8α silenced population displaying >5-fold reduced CD8α expression. The majority of cells within this population did not express GFP. However, cells that did express GFP also silenced CD8α. This corresponded to ~20% of the total cells, similar to the control GFP alone (Fig. 1*B*, NS siRNA, 24 h). This indicates that all of the cells expressing GFP also received an adequate level of siRNAs to silence CD8α. In addition, a large fraction of cells incorporated biologically active levels of siRNAs and

yet did not express plasmid DNA. In this experiment, time points were taken over a period of 6 days. At each time point, one-half of the cells were removed from the dish and replaced with fresh medium. The collected cells were stained for CD8α and analyzed by flow cytometry (Fig. 1). A decrease in CD8α surface expression was detectable at 12 h posttransfection, with maximal silencing at 36 h. By 96 h, nearly all of the cells expressed wild-type levels of CD8α. Thus, the RNAi effect in these T cells is a transient phenomena.

In these experiments, there was a dramatic decrease in GFP expression over time, which was likely a result of dilution of the plasmid or potentially due to toxicity of high GFP expression. Because 100% of the GFP-expressing cells exhibited CD8α silencing, it was possible to monitor the "fate" of this subset of silenced cells. The T cells that actively underwent CD8α silencing continued to express GFP over the time course, to the same level as the control population of cells that were not transfected with siRNAs (compare nonspecific RNA to CD8 siRNA). At 96 h, <5% of the total cells were GFP-positive in cells treated with nonspecific siRNAs and in CD8α siRNA-treated samples. These few remaining GFP-positive cells exhibited normal levels of CD8α expression. This suggests that the cells did not specifically undergo apoptosis as a result of siRNA transfection and subsequent CD8α silencing.

Specificity of siRNA-mediated silencing

Although the GFP transgene expression was not affected during CD8α silencing, the expression of endogenous genes might have been nonspecifically affected. To address this question, the expression levels of CD4 and Thy1.2 T cell markers were examined in cells actively undergoing CD8α silencing. Examination of these markers revealed that there was no reduction of nontargeted gene expression when compared with the control nontransfected cells (Fig. 2*A*), even over extended times (not shown). Although unlikely for this cell line, an additional analysis confirmed that the T cells did not become activated, as they do not up-regulate CD69 (Fig. 2*A*). Together, these experiments confirm the specificity of siRNA-mediated CD8α silencing.

Stability of targeted CD8α mRNA

Short temporal RNAs such as *lin-4* and *let-7* mediate silencing by binding to the 3′-untranslated region (UTR), thus suppressing translation (24–26). This is in marked contrast to the posttranscriptional mRNA degradation effected by siRNAs. To distinguish between these two potential mechanisms for CD8α silencing, a time course Northern blot analysis of CD8α mRNA was performed. The process of silencing did not appreciably affect the growth rate, as compared with control nonspecific siRNA transfections performed in parallel (not shown). Flow cytometry analysis indicated that the RNAi response in these cells lasted 3–4 days (8–10 cell doublings), which corresponds to an ~100-fold increase in cell mass (Fig. 2*B*). Time course analysis was performed in four independent experiments and expression of CD8α was typically suppressed ~5-fold or greater.

At various time points, a fraction of the cells was used to isolate total RNA for Northern blot analysis (Fig. 2*C*). The CD8α mRNA was resolved into two bands, due to alternative splicing (27, 28). Levels of CD8α mRNA decreased during the course of CD8α silencing. Densitometric analysis of the CD8α mRNA bands was performed and normalized to the internal control CD4 band. At the point of maximal silencing, mRNA levels decrease only 2.5-fold. This value is not commensurate with the ~5-fold decrease in protein expression determined by the flow cytometric analysis. However, this RNA was prepared from total cells in which 30% of the

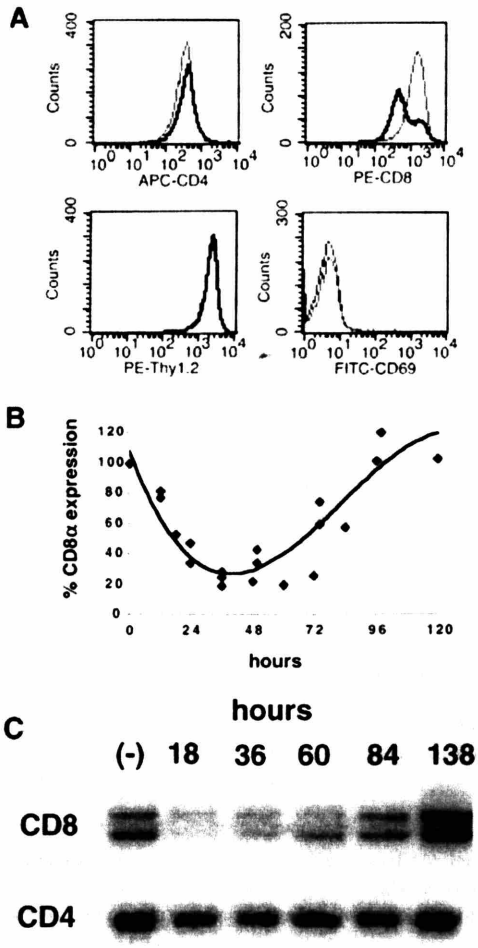

FIGURE 2. Characterization of CD8α siRNA-mediated silencing. *A*, CD8α siRNAs reduce the expression of CD8α, but do not affect the expression of CD4, Thy1.2, or CD69. E10 cells undergoing CD8α silencing at 36 h were immunostained and analyzed via flow cytometry. Histograms plot the number of cells (counts) vs the expression level of each marker (*bottom axis*). Bold line: with siRNA; thin line: without siRNA. *B*, Time course of CD8α silencing. E10 T cells were transfected with siRNAs and cultured over 10 days. During that time, cells were removed from the dish and flow cytometry was performed. Shown are collected data points from three independent experiments. Percent cells expressing normal levels of CD8α are plotted against the time (hours). *C*, Northern blot depicting CD8α mRNA during silencing. At different time points in *A*, RNA was harvested from the cells and probed for CD8α mRNA (the two bands correspond to alternative splicing of CD8). The blot was stripped and reprobed for CD4 mRNA as a loading control.

cells did not exhibit any silencing. When corrected for this reduction, CD8α mRNA was nearly proportionate to levels in reduction of CD8α protein. These Northern blots were performed multiple times with similar results. Thus, although it is clear that CD8α mRNA decreases, we cannot rule out additional silencing phenomena such as cotranslational repression.

Regional sensitivity of an mRNA to silencing by a siRNA

A major outstanding question is whether any region of a mRNA can serve as an effective target for siRNA-directed silencing. Several different siRNAs that targeted different regions of the CD8α

mRNA were tested. Of the first two CD8α siRNAs that were transfected, only one was active. To more quantitatively examine this difference, cells were transfected with varying amounts of siRNAs and CD8α expression was measured by flow cytometry. Cells undergoing silencing were quantified and compared with control nonspecific siRNA treatment (Fig. 3*A*). For the effective CD8α siRNA, picomolar amounts were sufficient to induce some silencing and higher amounts produced a graded response. For the noneffective CD8α siRNA, even at the highest concentration tested, there was no activity.

As these studies progressed, we observed that the majority of the synthetic CD4 and CD8α siRNAs were noneffective at silencing. For CD8α, four different siRNAs were synthesized and tested in the flow cytometry assay: one overlapped the start codon, one which targeted the open reading frame (ORF), one which overlapped the stop codon, and one which targeted the 3′-UTR 15 nt after the stop codon. Only the siRNA which targeted the 3′-UTR ~15 nt after the stop codon effectively silenced CD8α expression. For CD4, five siRNAs were synthesized which targeted corresponding regions to those for the CD8α mRNA (Fig. 3*B*). In this case, only the siRNA that targeted the stop codon was effective at reducing CD4 expression levels. An examination of the nucleotide sequences did not reveal any obvious differences between the effective and ineffective siRNAs.

For each of the above siRNAs, the silencing assay was performed at different siRNA concentrations. None of the inactive siRNAs generated detectable silencing at five times the highest concentration of the active siRNAs (Fig. 3*A* and data not shown). However, these inactive siRNAs were able to compete with the silencing of the active siRNAs. In these competition experiments, inactive CD8α siRNAs were added into the cuvettes containing the active CD8α siRNA, so that both could be electroporated into the cells simultaneously. Varying concentrations were tested, and cells were monitored for CD8α silencing at 36 h (Fig. 4). It was found that when the total siRNA pool contained an inactive CD4 or CD8α siRNA, then silencing mediated by an active siRNA was markedly reduced (Fig. 4, *A* and *B*). These results mirror the ability for active siRNAs to compete for other active siRNAs, a response that we observed for attempting silencing of both CD4 and CD8α simultaneously (Fig. 4, *C* and *D*). The inability to silence both CD4 and CD8α simultaneously in the same cell might suggest that siRNA-mediated RNAi is titratable, as has been described for silencing using long dsRNAs in *C. elegans* (29).

To test whether the above siRNAs were also inactive in other cell types, the CD4 and CD8α genes were expressed from CMV-driven promoters in HeLa cells. The CD8α expression construct contained two regions that corresponded to target sites for effective and ineffective siRNAs in E10. In this assay, cationic lipid cotransfection of the mouse CD4 and CD8α plasmid vectors was performed with either the effective or noneffective CD8α siRNA. When compared with the nonspecific siRNA control, CD8α-specific RNAi silencing was recapitulated in HeLa cells, and the ORF-targeted siRNA was still ineffective at silencing (Fig. 5*A*). These results suggested that the noneffective siRNA phenomenon is not unique to the T cell line, but is likely a feature of either the siRNA sequence, or more likely the mRNA. The concentration dependence of the effective and ineffective siRNA was evaluated in the HeLa cell assay. In this experiment, cationic lipid:siRNA complexes were preformed and added to the cells as previously described (13). The effective siRNA exhibited a concentration dependence; however, the ineffective siRNAs remained inactive even at the highest concentrations (Fig. 5*B*).

siRNA-mediated silencing in primary mouse T cells

To test whether primary cells are sensitive to siRNA-mediated silencing, the CD4/CD8α siRNAs characterized above were used to silence in primary mouse T cells taken from spleen. In these

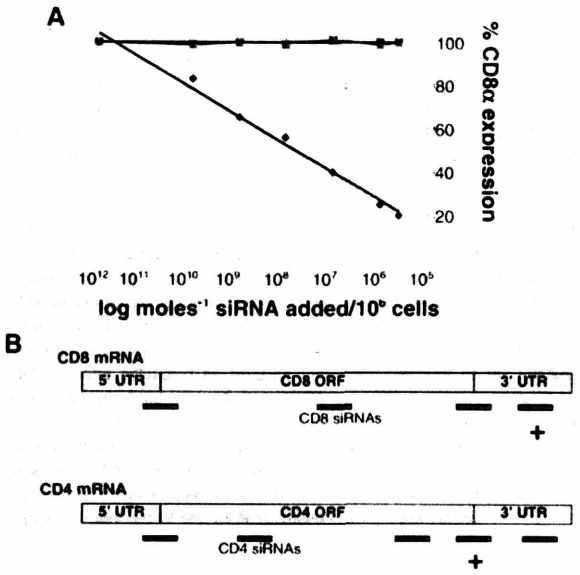

FIGURE 3. A restrictive number of designed siRNA sequences are effective at silencing. *A*, Effect of three different siRNAs targeting CD8α. Effective concentrations of three different CD8α siRNAs were evaluated by titrating increasing amounts of the siRNAs into the cuvettes before electroporation. CD8α expression was evaluated 36 h posttransfection, and is plotted as a percentage of wild-type levels. Effective siRNA (♦); two ineffective siRNAs (▲, ■). *B*, Relative target locations of the siRNAs used in these studies. Schematic of CD4 and CD8α mRNAs (not to scale), showing ORF and 5′- and 3′-UTR. +, Indicates siRNAs that are effective.

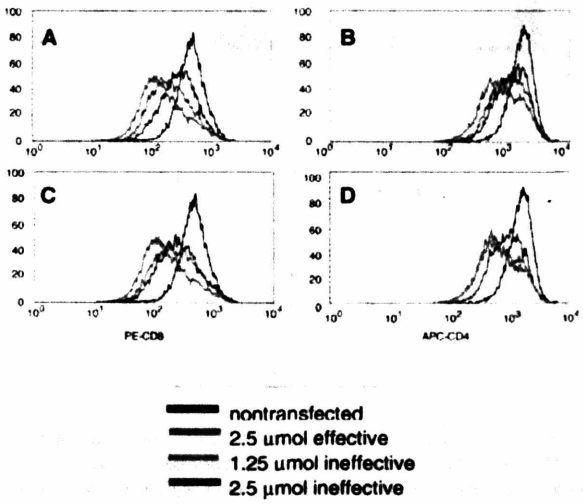

FIGURE 4. Effective and ineffective siRNAs can compete for silencing. Effective CD8α (*A*) and CD4 (*B*) siRNA-mediated silencing is competed by increasing concentration of cotransfected ineffective CD4 and CD8α siRNAs. Effective CD8α (*C*) or CD4 (*D*) siRNA-mediated silencing is competed by increasing concentrations of cotransfected ineffective CD8α or CD4 siRNA. Red indicates E10 cells transfected with 2.5 μmol effective siRNA and black is the nontransfected control. Green and blue indicate the addition of half the amount of an ineffective siRNA.

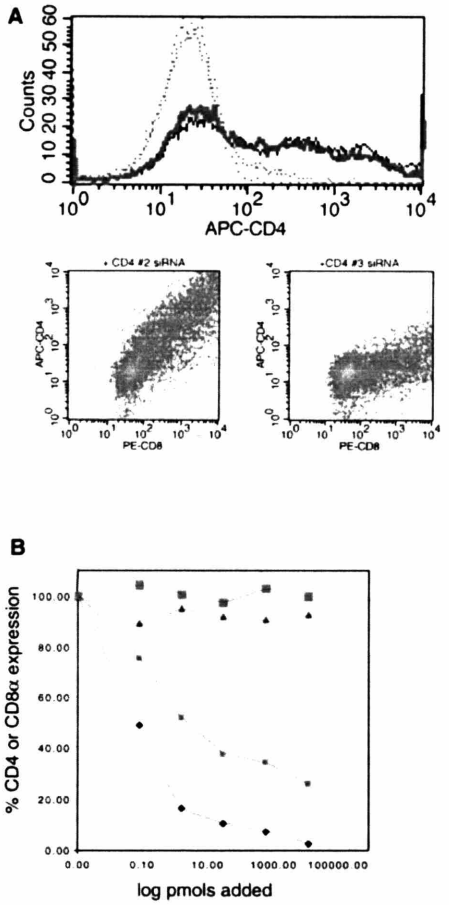

FIGURE 5. Restrictive siRNA usage is not a T cell specific phenomenon. *A*, CD4 silencing in HeLa cells. CD4 and CD8α expression vectors (with or without CD4 siRNAs) were transiently transfected into HeLa cells. The histogram at the top shows the distribution of transfected cells (counts) expressing CD4 (green), and cells that were cotransfected with effective (dotted pink) or ineffective CD4 siRNAs (black). The dotted blue line indicates nontransfected HeLa cells. The bottom two density plots show the specificity of CD4 silencing. CD4-silenced cells were stained for CD4 and CD8α markers. The lower left density plot depicts a typical expression profile of an ineffective siRNA, which is identical to nonspecific siRNA control (not shown). The lower right density plot shows typical results of CD4 silencing using the effective siRNA, which does not affect CD8α expression. *B*, Titration of CD4 or CD8α siRNAs into the HeLa cell system. Effective concentrations of two different CD4 and two different CD8α siRNAs were evaluated by titrating increasing amounts of the siRNAs during cationic lipid cotransfection. CD4 and CD8α expression was evaluated 36 h posttransfection. Effective CD8α siRNAs (green) and CD4 siRNAs (black) reduce CD4 and CD8α expression, while ineffective CD4 (blue) and CD8α (red) maintain high expression levels. Ordinate shows the expression of either CD4 or CD8, which is normalized to 100%. Abscissa depicts the amount of the siRNAs added during transfection.

studies DO11.10 mice, which express a transgenic TCR that recognizes OVA peptide in the context of MHC class II were isolated from these mice are predominantly $CD4^+$; however, a small number ($\sim$15%) of $CD8^+$ cells exist in these mice. Efforts to transfect and silence naive T cells were unsuccessful, but if the cells were stimulated to divide by the cognate OVA peptide, CD4 and CD8α silencing could be accomplished similar to the E10 thymoma cell line. Electroporation of CD4 siRNAs into activated primary T cells resulted in an approximate 5-fold decrease in CD4 surface expression compared with an unrelated siRNA control (Fig. 6). Costaining for CD8α on the same cells demonstrated that the down-regulation of CD4 was specific. The maximal degree of silencing was reached at 48 h posttransfection. Later time points could not be collected because of reduced cell viability after 72 h in culture. Similarly, the subset of CD8-positive T cells electroporated with CD8 siRNA exhibited a maximal 3.3-fold decrease in CD8α levels. Furthermore, the degree of silencing in the sample population with the alternate coreceptor (i.e., CD4 in a CD8α siRNA-treated sample) verified that the RNAi response was specific (data not shown). These results demonstrate that primary, mature T cells are able to perform RNAi. The overall degree, kinetics, and specificity of silencing of CD4 or CD8α in primary T cells was comparable to that of the E10 cell line, further supporting the validity of using this line to characterize T cell RNAi.

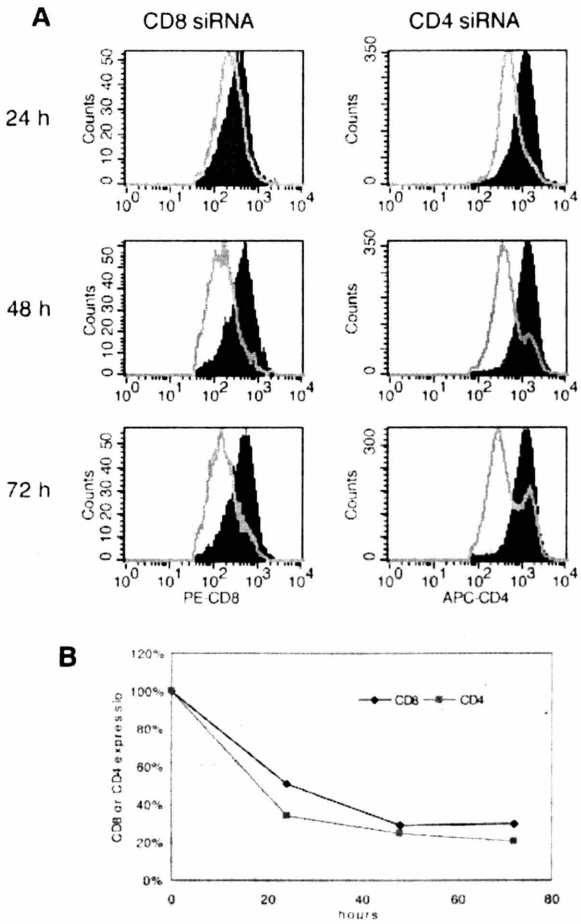

FIGURE 6. Time course of CD4 and CD8α suppression by siRNAs in primary T cells. *A*, Activated DO11 T cells were transfected with siRNAs and cultured for 3 days. Cells at each time point were analyzed by flow cytometry. The histograms are gated on viable cells that express either CD4 or CD8α respectively. The overlays (gray lines) in the histograms represent cells transfected with siRNAs specific for either CD4 or CD8; whereas the underlying histograms (filled) represent controls transfected with a nonspecific siRNA control. *B*, Time course of CD4 and CD8α silencing. The maximal level of suppression was determined by finding the peak fluorescence level of the suppressed curve and expressing it as a percent of the peak fluorescence level of nonspecific siRNA transfection control. Values are expressed as percent silencing and are plotted against time (hours).

Discussion

The CD4 and CD8α T cell surface glycoproteins are of central importance to immune function and disease. We have quantitatively tested the efficacy of a variety of siRNAs to suppress the expression of these glycoproteins. Targeting the CD4 and CD8α markers was attractive since turnover of coreceptor message is fairly rapid (~12 h for CD8α), and changes in surface expression can be rapidly and easily assayed by flow cytometry. In this analysis of two different genes, we observed that T cells and thymocytic cell lines are amenable to siRNA-mediated silencing. These studies revealed that siRNA-mediated RNAi is transient, lasting approximately eight cell doublings. Not every siRNA was able to induce silencing, and the RNAs which targeted the 3′-UTR were effective for both genes. Although small temporal RNAs (stRNAs) mediated translational repression at the mRNA 3′-UTR (for recent reviews, see Refs. 30–34), Northern blot analysis of CD4 and CD8α mRNA indicated posttranscriptional degradation of the mRNA, consistent with a RNAi-type mechanism of silencing. Finally, in primary T cells, the overall penetrance and kinetics of CD4 and CD8α siRNA-mediated RNAi was found to be similar to that observed in the E10 thymoma cell line.

In several experiments, and using electroporation, we found efficient uptake and silencing of >90% of the cells. However, this required the addition of a relatively high amount of siRNA (2.5 μmol/1.5 × 10^7 cells); Northern blot analysis indicates that only a fraction of the siRNAs (~3 × 10^4 siRNAs/cell) become associated with the cells (data not shown). Only a fraction of the siRNAs that become associated with cells probably are functional in silencing gene expression. At lower concentrations of siRNAs, a similar fraction (70–95%) of cells exhibit a reduction in CD8 expression, albeit at reduced efficiency. Using either electroporation for T cells or Lipofectamine 2000 for HeLa cells, we found that 100% of the cells that take up and express a cotransfected GFP marker also perform RNAi. Based on this fact, it should be possible to design gene function experiments which enrich the pool of silenced cells by selecting for the activity of a transfected plasmid reporter.

Time course analysis of CD8α silencing in the E10 cell line indicated that the silencing was transient in nature, lasting ~3–4 days. As this cell line doubles rapidly, this value corresponds to approximately eight cell doublings. Northern blots indicated that silencing corresponded to a reduction in mRNA levels, commensurate with the predicted model for RNAi. A translational repression mechanism has been suggested for silencing mediated by stRNAs via the 3′ untranslated region of developmentally important genes. Although the reduction in mRNA level approximated that of CD8α expression, we cannot rule out the possibility of additional translational repression mechanisms.

Only a limited number of the siRNA sequences tested could induce RNAi. For the silencing of most genes, on average one of two candidate siRNAs designed is active in contrast to the one of four and one in five siRNAs tested in targeting CD4 and CD8α (6). It is interesting to note that the siRNAs that were active in silencing targeted the 3′-UTR and stop codon. The restrictive utilization of the 3′-UTR siRNAs did not appear to be cell-type specific, as active and inactive siRNAs gave similar results in HeLa cells. It is unclear why targeting the mouse CD4 and CD8 mRNA 3′-UTRs were effective for performing siRNA-mediated RNAi, while other sites were not. One possibility is that further testing of other mRNA regions would result in productive silencing (35). Alternatively, perhaps the 3′-UTR of these genes is particularly accessible for targeting. Silencing of developmentally timed genes in the endogenous stRNA pathway is specific for the 3′-UTR (25, 36). This could be a common feature of developmentally timed genes, because both CD4 and CD8 are also expressed in a developmentally timed manner.

Attempting to silence both CD4 and CD8α simultaneously resulted in lower levels of silencing of each gene. These results supports a previously recognized observation that the RNAi response is titratable (29). Surprisingly, several of the siRNAs that were inactive competed for silencing when coelectroporated with active siRNAs. While this manuscript was in preparation, another group reported similar findings for the silencing of human coagulation trigger factor (37). However, another group has reported success in dual gene targeting of Lamin A/C and NuMA proteins in HeLa cells (38). The data presented in this study indicate that the inactive siRNAs are recognized by cellular processes but either cannot be converted to an active structure for gene silencing or cannot gain access to their complementary sequences on the target mRNA.

This work presents the first evidence for silencing by siRNA in primary somatic mammalian lymphocytes. In these studies, the degree and kinetics of CD4 and CD8α silencing in the activated primary cells was similar to that of the E10 cell line. In both the primary cells and E10 cells the onset of maximal silencing appeared around three to four cell doublings, which corresponded to 36–48 h posttransfection. In the E10 cells, 100% of the cells had resumed normal CD8α expression by 96 h. Because the viability of the primary cells began to diminish at around 60 h, it was difficult to determine how long the RNAi response would last past 72 h. It is interesting to note that the cells needed to be activated in order for silencing to be accomplished. This could be due to the inability to take up the siRNAs after electroporation, as primary T cells are known to be difficult to transfect with nucleic acids. It is unknown whether mammalian cells must be in a dividing, or "competent", state to perform RNAi. Future studies of siRNA-mediated RNAi in primary cells are required to distinguish between these two possibilities. Nevertheless, these findings provide a precedent upon which future studies of T lymphocyte biology can be designed to validate function by siRNA-mediated silencing.

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Appendix B

"Cloning of LentiLox Vectors."

Doug Rubinson.

The Lentilox series of vectors are the result of extensive modification of pBFGW.

Characterization of pBFGW

pBFGW is a 3rd generation lentiviral plasmid that was incompletely characterized and lacking sequence information. We sequenced this plasmid in its entirety. In short, pBFGW is based upon the pCDNA 3.1/Zeo plasmid (Invitrogen). Downstream of the pCDNA 3.1/Zeo CMV promoter was a cassette for the generation of lentivirus. This consisted of a 5' self-inactivating (SIN) LTR, the required packing sequence (Psi) , the HIV FLAP element (FLAP), a hybrid promoter consisting of beta-actin and CMV promoter sequences, the open reading frame for enhanced Green Fluorescent Protein (EGFP), the Woodchuck Hepatitis Regulatory Element (WRE), and the 3' SIN LTR. The intended use of pBFGW was for the introduction of transgenes and tissue-specific promoters for expression in non-dividing cells and embryos. However, there existed only three unique restriction sites for the introduction of transgenes and/or promoters. In addition pBFGW was 10,441bp without the introduction of a transgene. Plasmids of this size are more difficult to manipulate then smaller plasmids. There was also no mechanism to eliminate transgene expression after infection.

Generation of pLentiLox vectors

Elimination of elements between FLAP and WRE

pBFGW was serially digested with PacI and EcoRI. The 7,930bp fragment representing the backbone was purified by gel purification. The overhanging ends were filled-in by reaction with Pfu polymerase to generate blunt ends.

Introduction of a MCS-LoxP-MCS-LoxP

The plasmid, pBluescript Lox (pBS Lox) was digested with Asp718. A 209bp fragment was isolated by gel purification. This fragment contained a multiple cloning site (MCS), a LoxP site, a second multiple cloning site, and a LoxP site containing a three base pair deletion. This deletion was not identified until later in the construction of the LentiLox vectors (see below). The 209bp fragment was filled-in with cloned Pfu polymerase, and ligated into the 7,930bp fragment of pBFGW. The orientation of the insertion was determined both by restriction fragment length polymorphism and by sequencing. A plasmid containing the MCS-LoxP-MCS-LoxP with the expected sequence in the correct orientation was named pLentiLox 1.0.

Elimination of plasmid backbone restriction sites

Several of the sites found within the MCS of our MCS-LoxP-MCS-LoxP cassette were present elsewhere in the pLentiLox 1.0 vector. Specifically, NotI, ApaI, and XhoI cut both within the MCS cassette as well as each cutting once elsewhere in the vector. In order to gain the use of these sites we intended to destroy each site.

pLL1.0 was partially digested with NotI under condtions of limiting enzyme activity (.0625 Units of enzyme per microgram of pLL1.0 incubated at 37 degrees Celsius for twenty minutes). The 8,142bp band that represented linearized pLL1.0 was isolated via agarose gel electrophoresis followed by gel extraction. This linearized fragment was phosphorylated on its 5' ends with T4 Polynucleotide Kinase (PNK). The overhanging ends of this molecule were then filled-in with cloned Pfu to destroy the NotI site. The ends were ligated together to circularize the molecule. To determine whether a NotI site had been destroyed and to determine which NotI site had been destroyed the plasmid was digested simultaneously with NotI and with PstI. Destruction of the NotI site in the plasmid backbone yielded fragments of 7830bp and 312bp whereas destruction of the MCS NotI yielded fragments of 7253 and 889bp respectively. We accidentally chose a plasmid in which the MCS LTR was destroyed and named it pLentiLox 1.1 (pLL1.1). This was later fixed (see below). It should be noted that this step required several attempts to create. The pLL1.0 plasmid was prone to frequent recombination within the plasmid causing large deletions.

We designed a strategy to destroy ApaI that would also eliminate a 2197bp fragment between the 3'SIN LTR and the pUC ori that we deemed non-essential for lentiviral production. This ApaI-PciI fragment contained a BGH polyadenylation site, an SV40 promoter/ori, the Zeomycin resistance gene, and an SV40 polyadenylation site. We digested pLL1.1 with PciI to linearize the plasmid. The linearized plasmid was then digested with a limiting amount of ApaI (between .25 Units and 2 Units per microgram of linearized pLL1.1 for twenty minutes at room temperature). A 5,945bp fragment representing a single cut with ApaI adjacent to the 3' LTR was isolated by agarose gel electorpheresis followed by gel purification. The gel purified fragment was phosphorylated with PNK, filled in with cloned Pfu, and circularized by ligation. The ligated DNA was digested with with StuI prior to transformation into bacteria. Digestion with StuI should specifically cut plasmid that contains the 2197bp fragment we eliminated, and thus was used to select against contamination with uncut pLL1.1 vector. The elimination of the 2197bp and the destruction of PciI and ApaI was verified by restriction digest and a correct plasmid was identified and named pLentiLox 1.2 (pLL1.2).

To destroy the XhoI site, pLL1.2 was cut with limiting amounts of XhoI (.0625 Units per microgram of plasmid for twenty minutes at 37 degrees Celsius). A 5,947bp fragment representing single-cut linearized pLL1.2 was isolated via agarose gel electrophoresis and gel purification. The fragment was 5' phosphorylated with PNK, filled in with cloned Pfu, recircularized with ligase, and transformed into bacteria. Destruction of the correct XhoI site was verified by restriction digest. A correct plasmid was identified and named pLentiLox1.3(pLL1.3).

Expansion of the 5' MCS

The MCS in pLL1.3 adjacent to the FLAP sequence was intended for the insertion of promoter sequences. After destruction of the sites mentioned above we had two unique cloning sites in this MCS (ApaI and XhoI). We derived a list of restriction enzymes that failed to cut pLL1.3 to generate a list of candidate sites to engineer into an expanded 5'MCS. We then designed complimentary oligonucleotides to allow us to introduce

XbaI, HpaI, NheI, and PacI sites between the ApaI and XhoI sites. The oligos were designed to include two nucleotides between adjacent restriction sites. The sequence of the sense oligo was 5' cgctctagacggttaacgcgctagccgttaattaagcc 3'. The antisense oligo was complimentary to this sequence but contained an additional four nucleotides at the 5' end to produce an XhoI overhang and four nucleotides at the 3' end to produce an ApaI overhang. The antisense oligo sequence was 5' tcgaggcttaattaacggctagcgcgttaaccgtctagagcgggcc3'. We chose restriction sites to include based upon the following criteria: 1. Inclusion of a restriction enzyme that leaves a blunt end. 2. Inclusion of a restriction site that has an 8bp recognition sequence. 3. Enzymes are widely available. 4. Enzymes are known to be reliable cutters.

pLL1.3 was digested sequentially with ApaI and XhoI. The digest was then purified by Qiaquick PCR purification kit (Qiagen) to eliminate the small DNA fragment between ApaI and XhoI. The fragment was then treated with Shrimp Alkaline Phosphatase (SAP) to eliminate 5'-phosphate groups. The oligos described above were synthesized, 5' phosphorylated, and PAGE-purified by IDT Corp. (www.idtdna.com). 60 picomols of each oligo were annealed in annealing buffer (100mM Potassium Acetate, 30mM HEPES-KOH pH 7.4, 2mM Magnesium acetate) by incubation at 95 degrees for 4 minutes, followed by 70 degrees for 10 minutes, then slowly cooled (.1 degrees/second) to 4 degrees, then maintained at 4 degrees for 10 minutes. The annealed oligos were diluted and ligated at an equimolar concentration with the linearized pLL1.3 vector. A plasmid containing the engineered MCS was identified by restriction digest and named pLL1.4.

It was at this time that we first realized that we had accidentally destroyed the incorrect NotI site (see above). The second MCS site (adjacent to the LTR) was then destroyed in pLL1.4. pLL1.4 was digested with NotI to linearize the plasmid. The ends 5' phosphorylated with PNK and were filled-in with cloned Pfu to blunt and destroy the NotI site. The plasmid was recircularized by ligation and transformed. We checked for destruction of the NotI site by restriction digest and named this plasmid pLentiLox 1.5 (pLL1.5).

We next sought to expand the MCS that is located between the two LoxP sites (3' MCS). We designed primers to introduce NsiI, SphI, SmaI/XmaI, AscI, and BamHI sites between the NotI and EcoRI sites. We followed the same design criterion as described above. An additional criterion was that no three consecutive nucleotides would generate a nonsense codon. This would allow us to produce fusion proteins in which MCS sequence can remain between the fused proteins without having to worry about premature peptide termination. In addition we wanted a minimum of sites in the MCS to be present in EGFP and dsRed2 which we intended to include in many derivatives of our vectors (see below). We purposefully intended to make this MCS more versatile then the 5' MCS since we anticipated that most application of our vector would require cloning into this MCS. The inclusion of an SphI site was fortuitous. The two nucleotide spacer between the NsiI and SmaI/XmaI led to the creation of an SphI site that overlaps these other two sites. More fortuitously, SphI is a unique site in pLL1.5. The oligos are as follows:
3' MCS Sense: 5' ggccgccgatgcatgccccgggatggcgcgccatggatccgcg 3'
3' MCS Antisense: 5' aattcgcggatccatggcgcgccatcccggggcatgcatcggc 3'

Because we had destroyed the NotI site that should have been present in the pLL1.5 3' MCS we had to use a different strategy to insert the oligos then was used for

the 5' MCS. The pBS-Lox (described above) plasmid was digested with NotI and EcoRI enzymes. The small DNA fragment that was liberated was eliminated by purifying the linearize pBS-Lox backbone in a Qiaquick pcr purification kit. The DNA was SAP treated. The 3' MCS oligos were annealed (see above). 150fmols of annealed oligos and cut pLL1.5 were ligated together and transformed. A plasmid containing the expanded 3' MCS was identified by restriction digest and named pBS-Lox-MCS.

To insert the expanded MCS from pBS-Lox-MCS we replaced the EcoRI-XhoI fragment from pLL1.5 (containing the improperly destroyed NotI site) with the EcoRI-XhoI fragment from pBS-Lox-MCS (containing an intact NotI site and the expanded 3' MCS). A plasmid containing the expanded MCS and intact NotI in the pLL backbone was identified by restriction digest and was named pLentiLox2.0 (pLL2.0).

Production of useful pLL2.0 series vectors

We next looked to produce vectors that would be useful starting points for many potential uses of our lentiviral plasmids. There is enormous interest in the generation of fusion proteins in which a gene is fused with a fluorescent protein. In particular, EGFP and dsRed2 are fluorescent proteins that are well characterized and widely available.

pLL2.1 was engineered to include the EGFP open reading frame. The EGFP open reading frame was amplified from pEGFP-N1 (Clontech) to include a 5'NotI site and a 3' NsiI site. The oligos used were:
EGFP/5'NotI: 5'-cggcggccgcgccaccatggtgagcaagggc-3'
EGFP/3'NsiI: 5'-cgatgcatcttgtacagctcgtccatgccg-3'
The pcr product was isolated by agarose gel electorpheresis, gel purified, and cloned into the NotI and NsiI sites of pLL2.0 to create pLL2.1.

pLL2.2 was engineered to include the dsRed2 open reading frame. The dsRed2 open reading was amplified from pdsRed2-N1 (Clontech) to include a 5'NotI site and 3'NsiI site. The oligos used were:
dsRed2/5'NotI: 5'-cggcggccgcgccaccatggcctcctccgag-3'
dsRed2/3'NsiI: 5'-cgatgcatcaggaacaggtggtggcggccc-3'
The pcr product was isolated by agarose gel electorpheresis, gel purified, and cloned into the NotI and NsiI sites of pLL2.0 to create pLL2.2.

Because the pLentiLox series has a self-inactivating 5' LTR, the provirus has no endogenous 5' promoter activity. Therefore, it is necessary to include an internal promoter to drive transgene expression. This makes the system compatible with tissue-specific promoters. We chose to clone a ubiquitous and constitutive promoter into our vector to create a promoter that should be active in most eukaryotic cell types and in all the tissues of a mouse. The promoter we chose was the Ubiquitin C promoter (UbC). We first attempted to clone UbC by pcr amplification of the promoter from the pUB6/V5/His vector. However, the UbC sequence was not robustly amplified via pcr. As a second strategy we digested the pUB6/V5/His vector with BglII and HindIII which generates a fragment containing the UbC promoter. This fragment was isolated by agarose gel electrophoresis and gel extraction. The fragment was filled-in with cloned Pfu polymerase and 5' phosphorylated with PNK. This fragment was ligated into HpaI digested pLL2.0, pLL2.1, and pLL2.2 to generate pLL2.3, pLL2.4, and pLL2.5 respectively. These plasmids were verified by restriction digest to contain the proper

insert. pLL2.4 and pLL2.5 were transfected into 293.T cells and production of the correct fluorescent protein was verified by visualizing the transfected cells under an epifluorescent microscope 24 hours after transfection.

Modification of pLL2.0 for use in RNAi

The design criterion for an RNAi plasmid was the following. The plasmid must contain a polIII promoter to drive expression of the RNAi-inducing stem-loop, and the plasmid must contain a polII promoter to drive expression of EGFP as a reporter. Because we were concerned that the placement of a strong polII promoter near a polIII promoter might interfere with polIII function we chose to place the polII-EGFP cassette between LoxP sites. This would allow us to eliminate the polII promoter if we were failing to accumulate the stem-loop RNA.

We first inserted a cassette to drive expression of EGFP. A DNA fragment containing the CMV promoter upstream of the EGFP open reading frame was amplified from pEGFP-C1. The oligos engineered a 5' NotI site and 3' EcoRI site. The oligos used were:
5'CMV/NotI: 5'-cggcggccgcgtggataaccgtattaccgccatg-3'
3'EGFP/stop/EcoRI: 5' cggaattcctacttgtacagctcgtccatgccgag-3'
The pcr product was isolated by agarose gel electrophoresis and purified by gel extraction. The fragment was cloned into the NotI and EcoRI sites of pLL2.0 to create pLL2.6. This plasmid was tested by restriction digest and production of EGFP was verified by transfection into 293.T cells.

The insertion of the U6 promoter presented an additional challenge. We needed to introduce a cloning site for the introduction of RNAi sequences. The U6 promoter has required sequences up until the +1 transcriptional start site. Therefore, one cannot modify the sequences prior to –1 without incapacitating U6. We engineered the U6 promoter to introduce an HpaI site that cuts at the –1 position of U6. The first three nucleotides of the HpaI site are present in the wildtype U6. We had to alter the nucleotides at –1 to +2 in order to engineer an HpaI site. As a result the U6 is not functional when containing the HpaI site. However after digestion with HpaI and introduction of oligos to code for a stem-loop, those oligos can re-generate the wild-type 3'end of the U6 promoter thereby restoring transcriptional activity. We engineered oligos to add a 5' XbaI site to the U6 promoter and 3' HpaI, BstEII, and XhoI sites. We cloned the amplified pcr product from the pMU6 (Mouse U6 promoter and gene) plasmid and introduced the product into the XbaI and XhoI sites of pLL2.6. The oligos used were:
5' XbaI/U6: 5'-gctctagagatccgacgccgccatctctag-3'
3' XhoI/BstEII/HpaI/U6: 5'-gcctcgagggtcaccgcgcgttaacaaggcttttctccaaggg-3'
The resulting plasmid was verified by both restriction digest and by sequencing and was named pLL2.7.

Repair of LoxP and engineering of new restriction site

It was at this point that we recognized that the 3' LoxP site in the original pBS-Lox contained a three nucleotide deletion that rendered it unusable. We decided to fix

the 3' LoxP site in the pLL2.0 plasmid, and then use this plasmid backbone to clone in sequences from pLL2.1-pLL2.7. The repair of the LoxP site gave us an opportunity to engineer a new restriction site outside of the LoxP site (between the 3' LoxP and the WRE). This site would give our plasmid series even greater flexibility for engineering other additions such as IRES-GFP, or inducible expression systems.

Oligos were designed to amplify a fragment from pLL2.0 from the EcoRI site to a PflMI site located within WRE. The 5' oligo extended from the EcoRI site in the 3'MCS through the mutant LoxP site and into the region between the LoxP and the WRE. This oligo was designed to add the deleted nucleotides to the LoxP site and to create a PciI site immediately following the LoxP site. The amplified DNA was inserted into the pLL2.0 backbone digested with EcoRI and PflMI.

The oligos used were:

5'EcoRI/LoxFix/PciI: 5'-
gcgaattcgtcgagggacctaataacttcgtatagcatacattatacgaagttatacatgtttaagggttccgg-3'

3' PflM1/Rev: 5'-aaggagctgacaggtggtggcaatg-3'

A plasmid was checked by sequencing for the addition of a correct LoxP and PciI sites and named pLL3.0.

In order to generate the pLL3.1-pLL3.7 series from the pLL2.1-pLL2.7 series we cloned ApaI-EcoRI inserts of various sizes (2.1-917bp, 2.2-875bp, 2.3-1417bp, 2.4-2,138bp, 2.5-2096bp, 2.6-1519bp, and 2.7-1819bp) from the pLL2.1-pLL2.7 vectors into the 5,831bp ApaI-EcoRI backbone from pLL3.0 to create pLL3.1-pLL3.7. All plasmids were verified by restriction digest.

Generation of specific RNAi vectors

In order to generate variants of pLL3.7 to specifically knockdown various genes we inserted oligos that would generate stem-loop structures when expressed from a U6 promoter. A target sequence was first identified (as described by Tuschl) from a candidate gene. The structure of the target is $AAGN_{18}TT$. In which GN18 has between 40-60% GC content and does not contain any strings of nucleotides. We then ordered complimentary oligos with the following general form:

5' TGN18TTCAAGAGA(81NC)TTTTT(XhoI overhang).

Appendix C

"A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference."

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A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference

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RNA interference (RNAi) has recently emerged as a specific and efficient method to silence gene expression in mammalian cells either by transfection of short interfering RNAs (siRNAs; ref. 1) or, more recently, by transcription of short hairpin RNAs (shRNAs) from expression vectors and retroviruses[2–10]. But the resistance of important cell types to transduction by these approaches, both *in vitro* and *in vivo*[11], has limited the use of RNAi. Here we describe a lentiviral system for delivery of shRNAs into cycling and non-cycling mammalian cells, stem cells, zygotes and their differentiated progeny. We show that lentivirus-delivered shRNAs are capable of specific, highly stable and functional silencing of gene expression in a variety of cell types and also in transgenic mice. Our lentiviral vectors should permit rapid and efficient analysis of gene function in primary human and animal cells and tissues and generation of animals that show reduced expression of specific genes. They may also provide new approaches for gene therapy.

Rapid progress in sequencing genes and characterizing their expression patterns has resulted in a growing list of coding regions predicted to contribute to mammalian tissue function and the development of disease. Current approaches to study gene function, such as generating knockout mice, are time-consuming and expensive and cannot be applied to human tis-

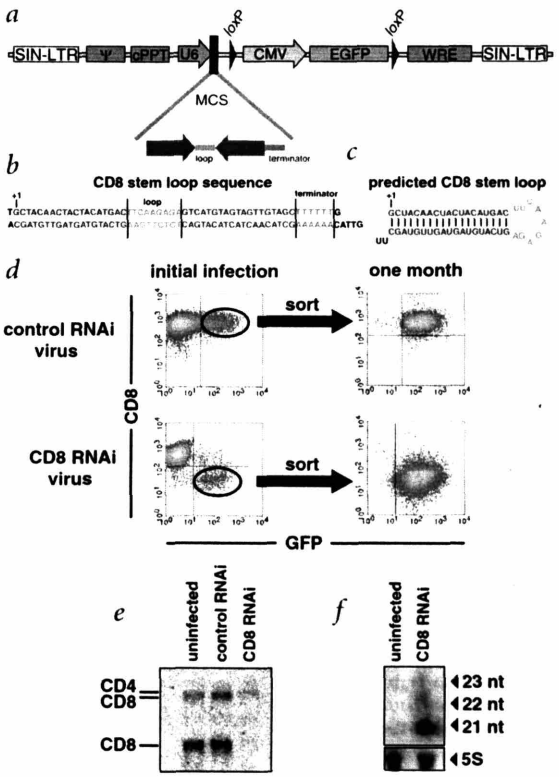

Fig. 1 Stable gene silencing and production of processed shRNAs in a T-cell line by a lentiviral vector. ***a***, Creation of an shRNA-expressing lentivirus vector. pLL3.7 was engineered by introducing the mouse U6 promoter upstream of a CMV–EGFP expression cassette to create a vector that simultaneously produces shRNAs and a reporter gene. To facilitate the introduction of RNAi stem-loops, a multiple cloning site was placed immediately after the U6 promoter. SIN-LTR, self-inactivating long terminal repeat; Ψ, HIV packaging signal; cPPT, central polypurine track; MCS, multiple cloning site; CMV, cytomegalovirus promoter; WRE, woodchuck hepatitis virus response element. ***b***, Sequence of the CD8 stem loop used in this study. A sequence known to silence CD8 as an siRNA[21] was adapted with a loop sequence from Brummelkamp *et al.*[3] to create an shRNA. The presumed transcription initiation site is indicated by a +1. Nucleotides that form the loop structure are indicated in green. The Pol III terminator stretch (a stretch of Us in the RNA) is indicated in red. ***c***, Predicted structure of CD8 shRNA produced from pLL3.7 CD8. ***d***, Stable silencing of CD8 by pLL3.7 CD8. The CD8 and GFP levels expressed by infected E10 cells 4 d after infection and after 1 mo of culture are shown. ***e***, Specific degradation of CD8 mRNA induced by pLL3.7 CD8. CD8 and CD4 mRNA levels in uninfected E10 cells, or E10 cells infected with either pLL3.7 (control virus) or pLL3.7 CD8 (CD8 RNAi virus) and sorted on the basis of GFP and CD8 expression, were assayed by northern blotting. The bands representing CD8 and CD4 mRNA species are indicated. ***f***, Generation of processed shRNAs in cells infected with pLL3.7 CD8. The cells analyzed for CD8 and CD4 mRNA levels in *e* were also examined for the presence of processed shRNAs by northern blotting. The location of RNAs of 21, 22 and 23 nt are indicated.

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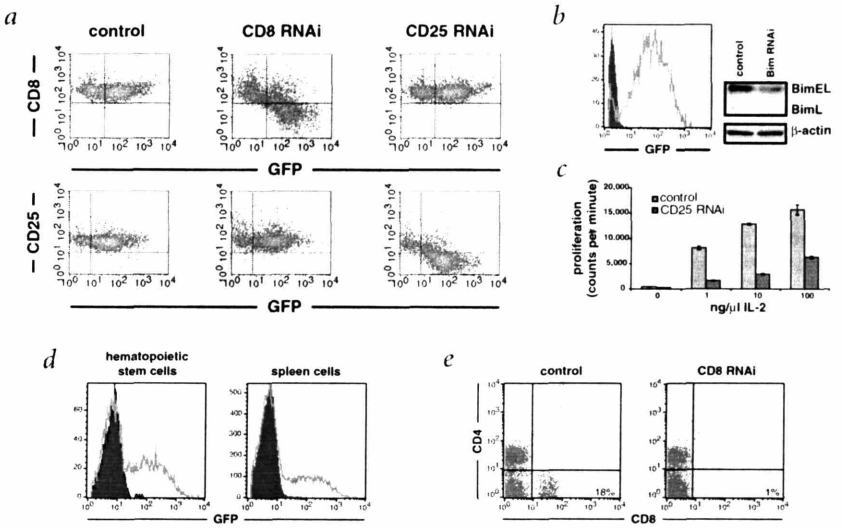

Fig. 2 Functional silencing of genes in cycling and non-cycling primary immune cells *in vitro* and *in vivo* by a lentiviral vector. ***a***, Specific silencing of genes in cycling T cells by pLL3.7 CD8 and pLL3.7 CD25. CD8-positive OTI T cells were activated with cognate peptide and then infected with pLL3.7, pLL3.7 CD8 or pLL3.7 CD25. The efficiency of infection was determined by assaying GFP expression. Expression of CD8 and CD25 in infected T cells was measured by staining with specific antibodies that bind these surface markers. ***b***, Efficient infection and gene silencing in non-cycling dendritic cells with pLL3.7 Bim. Bone marrow–derived dendritic cells were infected with pLL3.7 (data not shown) or pLL3.7 Bim. The efficiency of infection was determined by assaying GFP expression by flow cytometry (green line) and comparing with uninfected control cells (purple peak). Expression of Bim in pLL3.7 CD8 infected and control dendritic cells was assayed by western blotting. ***c***, Functional silencing of genes in primary T cells with pLL3.7 CD25. CD8-positive OTI T cells were infected and activated as in *a* and then cultured in the presence of increasing concentrations of IL-2. Proliferation was assessed by ^{3}H-thymidine incorporation. ***d***, Efficient infection of HSCs with RNAi lentiviruses and stable gene expression in differentiated progeny. HSCs were purified from the bone marrow of wild-type mice, infected with pLL3.7 (data not shown) or pLL3.7 CD8 and then cultured with IL-3, IL-6 and SCF. The efficiency of infection was determined by assaying GFP expression by flow cytometry (left histogram, green line) and compared with uninfected cells (purple peak). Bone marrow chimeras were generated by injecting sorted (GFP-positive) HSCs into lethally irradiated recipients. The contribution of these cells to the mature spleen cells of the chimeras was determined by staining with the congenic marker CD45.2, analyzing GFP expression by flow cytometry (right histogram, green line), and comparing with mice that received uninfected HSCs (purple peak). Histograms show GFP expression of HSC-derived (CD45.2-positive) cells. ***e***, Functional gene silencing in T cells derived from HSCs infected with pLL3.7 CD8. The percentage of CD8-positive T cells in the spleen of bone marrow chimeras from pLL3.7 (left dot plot) and pLL3.7 CD8 (right dot plot) was determined by staining with antibodies to CD4 and CD8, as well as the congenic marker CD45.2, and flow cytometry. Dot plots show CD4 and CD8 expression of HSC-derived (CD45.2) cells.

sues. RNAi has emerged as a rapid and efficient means to manipulate gene function in mammalian cells[12]. Retroviral vectors are efficient, stable gene delivery tools in mammalian cells[13,14], and recent studies suggest that they can stably express shRNAs in transformed and primary cells[10]. We have developed a lentivirus-based vector (pLL3.7) that expresses RNAi-inducing shRNAs under the control of the U6 promoter (Fig. 1*a*; ref. 15). This vector was engineered to co-express enhanced green fluorescent protein (EGFP) as a reporter gene, permitting infected cells to be tracked by flow cytometry (Fig. 1*a*,*d*). Lentiviruses have two key advantages over other gene delivery systems. First, they can infect non-cycling and post-mitotic cells[16,17]. Second, transgenes expressed from lentiviruses are not silenced during development and can be used to generate transgenic animals through infection of embryonic stem (ES) cells or embryos[18,19].

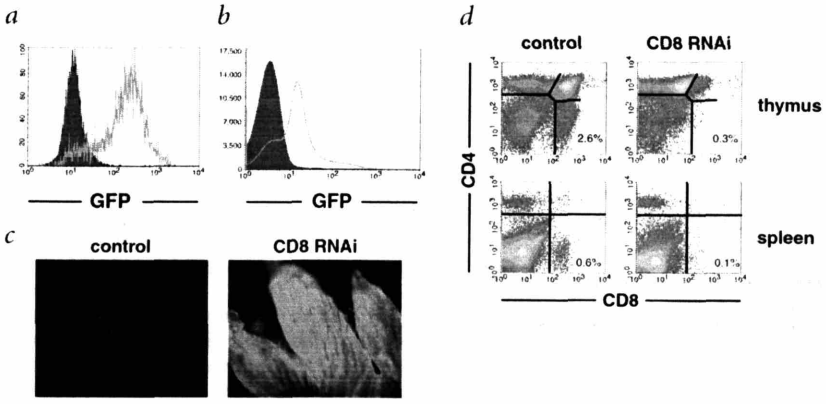

Fig. 3 Functional silencing of genes in ES cell–derived mice by a lentiviral vector. ***a***, Generation of stably infected ES cell lines with pLL3.7 CD8. AK7 ES cells were infected with pLL3.7 CD8 and sorted for GFP expression. GFP expression in cultured cells was determined 2 wk later by flow cytometry (green line) and compared with uninfected controls (purple peak). ***b***, Identification of ES cell-derived thymocytes in chimeric mice infected with pLL3.7 CD8. Thymocytes from uninfected (purple peak) and pLL3.7 CD8–infected (green line) ES-derived mice were harvested and analyzed for GFP expression. ***c***, Fluorescence imaging of paws of ES cell–derived mice infected with pLL3.7 CD8. The paws of control and pLL3.7 CD8–infected ES chimeric mice were imaged with standard epifluorescence for expression of EGFP. ***d***, Silencing of CD8 in the thymus and spleen of ES cell–derived mice infected with pLL3.7 CD8. Thymocytes and splenocytes from 1-wk-old control and CD8 RNAi (pLL3.7 CD8) ES cell–derived mice were harvested and stained for CD4 and CD8 expression.

Initial experiments indicated that pLL3.7 vectors could generate high-titre infectious lentiviruses that expressed shRNAs and silenced gene expression upon infection of mammalian cell lines (Fig. 1*d–f*). We next tested whether pLL3.7 could silence gene expression in primary mammalian cells by infecting OVA-specific CD8-positive OTI T cells derived from T-cell receptor transgenic mice[20]. T cells were activated with cognate peptide *in vitro* and infected with a version of pLL3.7 engineered to express an shRNA that silences expression of mouse CD8α (pLL3.7 CD8; Fig. 1*b*,*c*; refs. 4,21). Flow cytometric analysis showed that 68–82% of the cells were infected and reproducibly

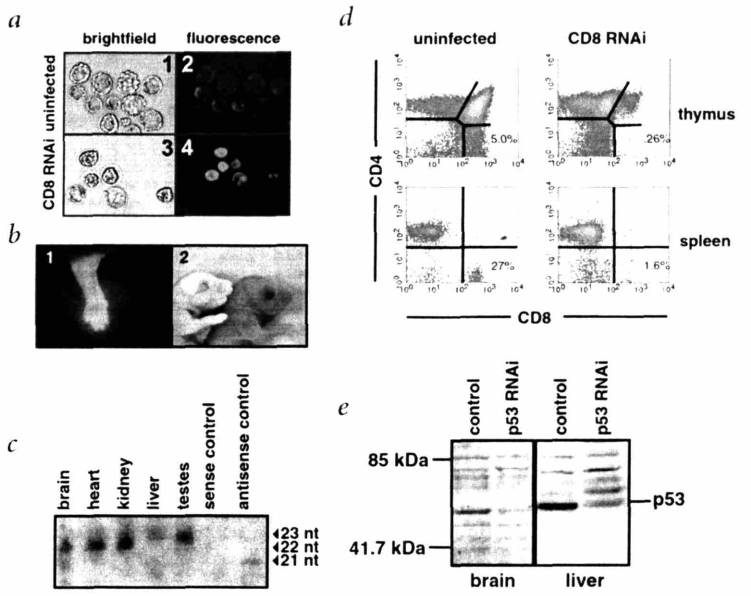

Fig. 4 Functional silencing of genes in transgenic mice derived from lentivirus-injected zygotes. **a**, Infection of zygotes with pLL3.7 CD8. Single-cell embryos were infected with CD8 RNAi (pLL3.7 CD8) or left uninfected and cultured for 3 d, at which time they were imaged with standard epifluorescence for expression of EGFP. ***b***, Generation of transgenic mice from pLL3.7 CD8–injected zygotes. Paw (panel 1) and whole mice (panel 2) derived from embryos that were uninfected (panel 2, right) and infected with pLL3.7 CD8 (panel 2, left) were imaged with standard epifluorescence for expression of GFP. ***c***, Expression of processed shRNAs in multiple tissues in transgenic mice derived from pLL3.7 CD8–injected zygotes. Tissues were harvested from 8-wk-old CD8 RNAi transgenic mice (pLL3.7 CD8) and assayed for the presence of processed shRNAs by northern blotting. ***d***, Silencing of CD8 in the thymus and spleen of transgenic mice derived from pLL3.7 CD8–injected zygotes. Thymocytes and splenocytes from 2-wk-old control and CD8 RNAi transgenic (pLL3.7 CD8) mice were harvested and stained for Thy1, CD4 and CD8 expression. Dot plots show the expression of CD4 and CD8 on T cells (Thy1-positive). ***e***, Silencing of p53 in the brain and liver of transgenic mice derived from pLL3.7 p53–injected zygotes. Brain and liver were harvested from 8-wk-old p53 RNAi transgenic mice (pLL3.7 p53) and control mice. The levels of p53 in these tissues were determined by western blotting.

showed approximately 93% lower expression of CD8 (Fig. 2*a*), indicating that lentivirus-driven expression of shRNAs efficiently silenced gene expression in primary T cells. This effect of pLL3.7 CD8 was specific, as infected T cells showed normal expression of other T-cell surface markers (Fig. 2*a* and data not shown). To determine whether pLL3.7-based vectors could also silence gene expression in non-cycling cells, we infected dendritic cells with RNAi lentiviruses that expressed shRNAs against the pro-apoptotic molecule Bim (ref. 22; pLL3.7 Bim). Infection of these cells was efficient, as gauged by expression of green fluorescent protein (GFP), and resulted in a reduction of Bim expression (Fig. 2*b*).

To determine whether lentivirus-mediated expression of shRNAs could induce functional silencing of a gene in primary cells, we infected OTI T cells with an RNAi lentivirus that targeted CD25, the IL-2 receptor α-chain (pLL3.7 CD25; Fig. 2*a*). IL-2 is an important growth factor for T cells, and T cells derived from mice that lack CD25 do not proliferate *in vitro*[23]. Activated OTI T cells infected with pLL3.7 CD25 showed a 95% reduction in IL-2Rα chain expression, but expressed normal levels of other surface markers (Fig. 2*a* and data not shown). These cells showed a 75–80% reduction in proliferation when challenged with increasing concentrations of IL-2 (Fig. 2*c*). As not all cells were infected in these experiments (typically 70–85% of cells were GFP-positive), the actual inhibition of IL-2–induced proliferation by RNAi was probably even more significant.

Lentivirus-based vectors are capable of stably expressing transgenes in stem cells and are not silenced during development[14,18]. We tested whether pLL3.7 could be used to silence gene expression in hematopoietic stem cells (HSCs) and their progeny. HSCs purified from whole bone marrow by cell sorting were infected with pLL3.7 or pLL3.7 CD8 and then cultured for 2 days in the presence of cytokines. This protocol led to infection of 30–60% of HSCs (Fig. 2*d* and data not shown). Next, GFP-positive cells were sorted and injected into lethally irradiated congenic mice. After 8 weeks, the injected HSCs had contributed to all blood cell lineages in reconstituted mice, as determined by staining for the congenic CD45 allele, and about 20–40% of HSC-derived lymphocytes were GFP-positive (Fig. 2*d*). The presence of GFP-negative cells is probably the consequence of the low activity of the CMV promoter in these cells[24]. To examine whether lentivirus-mediated expression of shRNAs in T cells resulted in gene silencing *in vivo*, we analyzed splenocytes from reconstituted mice. Mice that received HSCs infected with pLL3.7 CD8 showed at least a 90% reduction in the frequency of CD8+ T cells when compared with those receiving HSCs infected with pLL3.7 (Fig. 2*e*). No effect was seen on CD4-positive T cell levels and other immune cell populations, indicating that lentivirus-induced gene silencing was both functional and specific. We serially passaged bone marrow cells from reconstituted mice and still observed lentivirus-driven expression of GFP in hematopoietic cells (data not shown), confirming that we had infected true stem cells.

Lentiviruses have also been reported to infect mouse ES cells and to maintain their expression in transgenic mice generated from these cells[18]. We generated stable lines of ES cells that were infected with pLL3.7 CD8 or with versions of pLL3.7 that expressed shRNAs against p53 (ref. 6; pLL3.7 p53) or a neuron-specific isoform of Mena (ref. 25; pLL3.7 Mena+; Fig. 3*a* and

Table 1 • Efficient generation of RNAi transgenic mice through infection of zygotes with lentiviruses

RNA lentivirus	Litters	Total mice born	Number of transgenic offspring (percentage)	Average number of lentiviral integrants (range)
Control	2	15	8 (53%)	3.4 (2–5)
CD8	4	32	16 (50%)	3.1 (2–6)
CD25	7	42	11 (26%)	nd
P53	4	22	5 (23%)	nd

RNAi transgenic mice were generated with pLL3.7 (control), pLL3.7 CD8, pLL3.7 CD25 and pLL3.7 p53. Transgenic offspring were identified on the basis of their expression of GFP in the skin at 2–4 d. The number of lentiviral integrants present in the genome of transgenic (GFP-positive) mice was determined by Southern blotting. No lentiviral integrants were detected in five non-transgenic (GFP-negative) mice analyzed. nd, not determined.

Table 2 • Stable and functional silencing in adult RNAi transgenic mice

Age of RNAi transgenic mice (wk)	2	8	8	8	8	8	9	9
Percent decrease in CD8 expression	93	93	93	88	87	92	94	94
Decrease in percentage of mature CD8-positive cells	56	20	35	44	99	100	46	56
Number of lentiviral integrants	nd	2	3	2	6	5	4	3

The frequency of CD8-positive T cells present in neonatal and adult CD8 RNAi transgenic mice and age-matched controls, and the reduction of CD8 expression in cells showing gene silencing, were determined by staining lymphocytes with antibodies to Thy1, CD4 and CD8. The percent reduction in CD8-positive T cells seen in CD8 RNAi transgenic mice was determined by dividing the percentage of splenic T cells that were CD8-positive in CD8 RNAi transgenic mice by the percentage observed in control mice. The percent reduction in CD8 expression was determined by dividing the mean fluorescence intensity of CD8 staining in T cells (Thy1-positive) from CD8 RNAi transgenic mice that showed silencing by the mean fluorescence intensity of CD8 on T cells from control mice. The number of lentiviral integrants present in the genomes of the CD8 RNAi transgenic mice analyzed was determined by Southern blotting. nd, not determined.

data not shown). To test whether gene silencing could be maintained throughout organogenesis, we purified GFP-positive ES cell populations by cell sorting and injected these cells into RAG-deficient blastocysts. Because RAG deficiency blocks T and B lymphocyte development in the bone marrow, any peripheral T and B lymphocytes present in the chimeric progeny must be derived from the injected (RAG-positive) ES cells[26]. The degree of chimerism in animals derived from infected ES cells varied between 50% and 90%, as gauged by GFP fluorescence of whole mice and dissected organs (Fig. 3*c* and data not shown). About 20–40% of immune cells in these mice were GFP-positive (Fig. 3*b* and data not shown). To examine whether lentivirus-mediated expression of shRNAs resulted in the silencing of CD8 *in vivo*, we stained thymus and spleen cells of 7-day-old chimeric mice with antibodies to CD8 and CD4 and analyzed them by flow cytometry. Developing T cells in the thymus of pLL3.7 CD8–infected mice showed an 89% reduction in CD8 expression (Fig. 3*d*). In addition, we detected few mature CD8-positive T cells in this organ or in the spleen (Fig. 3*d*). By contrast, these mice showed normal expression of CD4 on thymocytes and normal percentages of mature CD4-positive T cells in immune tissues (Fig. 3*d*). No effects were observed on T-cell differentiation and frequencies in mice derived from ES cells infected with pLL3.7 Mena+ and pLL3.7 (Fig. 3*d* and data not shown).

Direct lentiviral infection of single-cell embryos provides an efficient and broadly applicable approach to generate transgenic animals[19]. We infected single-cell embryos with RNAi lentiviruses to determine whether this methodology could be used to bypass the use of ES cells. Embryos infected with pLL3.7, pLL3.7 CD8, pLL3.7 CD25, pLL3.7 p53 or pLL3.7 Mena+ expressed GFP after 3 days in culture (Fig. 4*a* and data not shown) and produced offspring that showed expression of GFP and siRNAs in all tissues tested (Table 1, Fig. 4*b*,*c* and data not shown). As in ES cell chimeras, 2-week-old transgenic mice generated from pLL3.7 CD8–infected zygotes showed a 91% reduction in CD8 expression in developing thymocytes (Table 2 and Fig. 4*d*). The frequency of mature CD8-positive T cells in the peripheral lymphoid organs of these mice was also reduced (Table 2 and Fig. 4*d*). Notably, gene silencing was maintained in adult mice (Table 2). As in HSC-reconstituted and ES cell chimeric mice, the expression of GFP in immune cells of RNAi transgenic mice generated with pLL3.7 vectors was low (5–60%). In all mice, cells exhibiting silencing showed an equal reduction of CD8 gene expression, but the percentage of cells showing silencing differed between transgenic strains (Table 2). This variation may be a function of the number of integrated lentiviral genomes present in different transgenic lines (Table 2). Gene silencing was not restricted to immune cells; brain and liver cells derived from transgenic mice that expressed pLL3.7 p53 showed reduced expression of the p53 tumor-suppressor protein (Fig. 4*e*).

The promise of RNAi to efficiently silence genes in mammalian cells is widely recognized[12,15,27,28]. The results presented here show that lentiviruses can be used to deliver shRNAs and reduce gene expression in cycling and non-cycling cells, as well as in chimeric and transgenic mice. This technology should allow systematic genetic analysis in most cell types and tissues, including those of human origin, and facilitate comprehensive studies of gene function in mice and in species that are not traditionally amenable to genetic manipulation. Lentiviral expression vectors might be used therapeutically to silence disease-causing genes, to render cells resistant to infectious organisms, and to facilitate the creation of tissues deficient in specific antigens as a source of transplant organs. Future modifications to lentiviral expression vectors, such as the inclusion of inducible or tissue-specific promoters, will extend the range of cells and situations in which they can induce RNAi.

Methods

RNAi lentivirus system. The complete details of the construction of pLL3.7 and other LentiLox vectors are described online (see URL). In brief, the pBFGW plasmid[19] was extensively modified to carry *lox*P sites, a CMV promoter driving expression of EGFP and the mouse U6 promoter with downstream restriction sites (*Hpa*I and *Xho*I) to allow the efficient introduction of oligonucleotides encoding shRNAs (Fig. 1*a*,*b*,*c*).

To confirm that pLL3.7 could silence gene expression in mammalian cells, we used an shRNA predicted to target the α-chain of CD8 to generate pLL3.7 CD8 (Fig. 1*b*,*c*). The CD8 shRNA was based on previously published sequences[4,21]. We generated lentivirus particles (as described below) and used them to infect E10 cells. Infected (GFP-positive) cells showed on average a 94% reduction of CD8 expression (Fig. 1*d*). Inhibition of CD8 expression was specific, as the levels of other surface proteins were not altered (Fig. 1*d* and data not shown). Moreover, in a subline of E10 cells engineered to express human CD8, which differs from mouse CD8 by 4 of 19 nt in the targeted region, only the mouse gene was silenced (data not shown). Cells infected with control virus (pLL3.7) or viruses expressing shRNAs against other genes showed no decrease in CD8 levels (Fig. 1*d* and data not shown).

To confirm that the decrease in surface expression of CD8 seen in infected E10 cells resulted from mRNA degradation, CD8 transcript levels in sorted (GFP-positive) cell populations infected with either pLL3.7 CD8 or a control virus were quantified by northern blotting (Fig. 1*e*). E10 cells that showed silencing expressed short RNAs of approximately 21 nt that were complimentary to an anti-sense strand of the CD8 stem loop, indicating that lentivirus-encoded shRNAs were being expressed and processed into siRNAs (Fig. 1*f*). To test the stability of lentivirus-induced RNAi in mammalian cells, we followed expression of CD8 in long-term cultures of E10 cells infected with pLL3.7 or pLL3.7 CD8 and sorted for expression of GFP. E10 cells infected with pLL3.7 CD8 virus were sorted 4 d after infection for GFP expression and low CD8 expression, whereas control virus infected cells were sorted for GFP expression only (Fig. 1*d*). Each population was cultured for 1 mo and analyzed for CD8 expression by flow cytometry at weekly intervals. We saw no change in expression of this surface receptor over the course of 1 mo, and these cells remained uniformly GFP-positive (Fig. 1*d*). But in each experiment a small fraction (2–10%) of infected (GFP-positive) E10 cells showed no evidence of gene silencing. These cells were shown to express no shRNAs (data not shown), suggesting that the activity of the U6 promoter was reduced, possibly owing to positional effects on the inserted transgenes[13,19].

shRNAs. The algorithm used to predict sequences that would lead to silencing of target genes, as well as primer sequences used for the construction of pLL3.7 and to create shRNAs against CD8, CD25, Bim, p53 and Mena+, are available online (see URL).

Generation and titre of lentivirus. Lentiviral production was done as described[19]. Briefly, we co-transfected pLL3.7 and packaging vectors into 293T cells and collected the resulting supernatant after 36 h. We recovered virus after ultracentrifugation for 1.5 h at 25,000 r.p.m. in a Beckman SW28 rotor and resuspension in phosphate-buffered saline (15–200 μl). Titers were determined by infecting 3T3 cells with serial dilutions of concentrated lentivirus. We determined GFP expression of infected cells by flow cytometry 48 h after infection; for a typical preparation, the titre was approximately $4–10 \times 10^8$ infectious units (IFU) per ml.

T cell and dendritic cell infection. CD8-positive T cells were activated by culturing splenocytes derived from OTI TCR transgenic mice at a density of 2×10^6 cells ml^{-1} in the presence of 1 μg ml^{-1} of OVA peptide and 100 ng ml^{-1} IL-2 (Biosource International). After 24 h and 48 h, cultures were supplemented with $20–100 \times 10^6$ lentiviral particles (multiplicity of infection (MOI) of 10–50), 10 μg ml^{-1} Polybrene, and spun at 1,200 r.p.m. for 1 h at 30 °C in a Beckman Allegra 6R centrifuge. Supernatant was removed after infection and replaced with growth medium containing 1 μg ml^{-1} OVA and 100 ng ml^{-1} IL-2. We collected T cells for experiments after 72 h. We generated dendritic cells by culturing whole bone marrow cells for 7 d at a density of 2×10^6 cells ml^{-1} in the presence of 20 ng ml^{-1} GM-CSF (Biosource International). During this culture period, fresh cytokine was provided every other day. The resulting cell populations were typically greater than 60% CD11c-positive as determined by flow cytometry (data not shown). On day 7, we transferred dendritic cells to a new plate, cultured them at a density of 2×10^6 cells ml^{-1} with medium containing no GM-CSF and infected them on day 8 and 9 with approximately 100×10^6 (MOI of 50) lentiviral particles. Cells were collected for analysis on day 10.

Hematopoietic stem cell infection. Whole mouse bone marrow cells were depleted for cells expressing B cell (B220) and granulocyte lineage markers (CD11b) using magnetic beads (Miltenyi), and then sorted for Sca1-positive and c-Kit-positive cells. These cells were spin-infected with RNAi lentiviruses at an MOI of approximately 10–50, and then cultured at a density of 2×10^6 cells ml^{-1} for 2 d in the presence of IL-3 (20 ng ml^{-1}, Biosource International), IL-6 (50 ng ml^{-1}, Biosource International) and SCF (50 ng ml^{-1}, Peprotech). We sorted infected HSCs for GFP expression, after which we injected approximately 2×10^5 cells into lethally γ-irradiated (1200 rads) recipient mice that were congenic for the CD45 antigen, to allow us to distinguish host and donor cells (HSCs were derived from a mouse strain that expresses CD45.2 on hematopoietic stem cells; recipient mice expressed CD45.1 on these cells). Most reconstituted mice also received 2×10^6 host-derived (CD45.1) whole bone marrow cells. Bone marrow chimeras were analyzed between 6 wk and 8 wk after reconstitution for GFP expression in splenocytes and CD8 gene silencing by staining and flow cytometry. In all experiments, progeny of infected HSCs were identified by staining for CD45.2.

ES cell infection. We maintained and infected AK7 ES cells as described[18]. Clones of ES cells were picked, expanded and analyzed by flow cytometry for GFP expression. If the clone contained a mixed population of infected and uninfected cells, we purified the GFP-positive population by fluorescence-activated cell sorting before blastocyst injection.

Generation of RNAi chimeric and transgenic mice. For ES cell–derived mice, we injected approximately 10–12 pLL3.7-infected GFP-positive ES cells into *Rag2*$^{-/-}$ blastocysts, which we then implanted into a pseudopregnant female recipient mouse[26]. We screened neonates resulting from these injections for chimerism by determining the level of GFP fluorescence of their skin and paws. Highly chimeric (>50%) neonates were used for analysis. To generate lentiviral transgenics, we injected a small volume of high-titre RNAi lentivirus ($4–10 \times 10^8$ IU μl^{-1}) into the perivitelline space of single-cell mouse embryos, which we then implanted into pseudopregnant female recipient mice[19]. The resulting neonates were screened for lentiviral integration by Southern blotting and expression by GFP fluorescence.

Flow cytometry. The following phycoerythrin-conjugated antibodies were used for flow cytometric analysis: antibody against CD4 (clone RM4-5), antibody against CD8α (clone 53-6.7), antibody against CD25 (clone PC81), antibody against CD45.2, antibody against CD95.2 (Thy1.2) and strepavidin. We also used allophycocyanin-conjugated antibody against CD8α and biotin-conjugated antibody against Thy1.2 for analysis. All antibodies were from BD Pharmingen. All plots shown are gated for viable cells, which were isolated by selecting PI^- cells.

Northern-blot analysis. For northern-blot analysis, we lysed cells with Trizol reagent (Invitrogen) and prepared total cellular RNA according to the manufacturer's instructions. RNA was prepared from tissues using RNAlater (Ambion Diagnostics) according to the manufacturer's instructions. We carried out CD4/CD8 probe hybridization as described[4]. For the small RNA northern blot, total RNA (60 μg) was fractionated on a 10% denaturing polyacrylamide gel and transferred to a nylon membrane. The membrane was hybridized to a probe consisting of a 21-nt CD8 siRNA sense strand labeled at the 5′ end with ^{32}P. We used a 5′ radio-labeled oligonucleotide probe to 5S RNA to determine equal loading of RNA.

Western-blot analysis. Mouse tissues were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate) supplemented with Complete Protease Inhibitor Tablets (Roche). We determined protein concentrations using BCA Protein Assay (Pierce). Equal amounts of protein (100 μg) were loaded per lane and separated on a 10% SDS–PAGE gel. Protein was transferred to a PVDF membrane. We detected p53 using antibody against p53 Ab-3 (Oncogene Research Products) diluted 1:1,000 and donkey antibody against mouse conjugated with horseradish peroxidase diluted 1:10,000. The blot was developed with ECL+ reagent (Amersham Biosciences).

Animal care. We carried out all mouse experiments according to the guidelines established by the Committee on Animal Care at the Massachusetts Institute of Technology or the Institutional Animal Care and Use Committee at Biogen.

URL. Additional information on pLL3.7 construction, target sequences used and target sequence selection can be found at http://web.mit.edu/ccrhq/vanparijs/.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Appendix D

"Cre-lox-regulated conditional RNA interference from transgenes."

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Cre-lox-regulated conditional RNA interference from transgenes

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We have generated two lentiviral vectors for conditional, Cre-lox-regulated, RNA interference. One vector allows for conditional activation, whereas the other permits conditional inactivation of short hairpin RNA (shRNA) expression. The former is based on a strategy in which the mouse U6 promoter has been modified by including a hybrid between a LoxP site and a TATA box. The ability to efficiently control shRNA expression by using these vectors was shown in cell-based experiments by knocking down p53, nucleophosmin and DNA methyltransferase 1. We also demonstrate the usefulness of this approach to achieve conditional, tissue-specific RNA interference in Cre-expressing transgenic mice. Combined with the growing array of Cre expression strategies, these vectors allow spatial and temporal control of shRNA expression *in vivo* and should facilitate functional genetic analysis in mammals.

RNA interference (RNAi) has emerged as a powerful tool to silence gene expression, and has rapidly transformed gene function studies across phyla. RNAi operates through an evolutionarily conserved pathway that is initiated by double-stranded RNA (dsRNA; for review, see refs. 1 and 2). In model eukaryotes such as plants and worms, long dsRNA (e.g., 1,000 bp) introduced into cells is processed by the dsRNA endoribonuclease Dicer into ≈21-nt small-interfering RNAs (siRNAs). siRNAs in turn associate with an RNAi-induced silencing complex and direct the destruction of mRNA complementary to one strand of the siRNA. Although the Dicer pathway is highly conserved, introduction of long dsRNA (>30 bp) into mammalian cells results in the activation of antiviral pathways, leading to nonspecific inhibition of translation and cytotoxic responses (3). The use of synthetic siRNAs to transiently down-modulate target genes, is one way to circumvent the cytotoxic dsRNA-activated pathways in mammals (4).

An important advance in the RNAi field was the discovery that plasmid-based RNAi can substitute for synthetic siRNAs, thus permitting the stable silencing of gene expression (5). In such systems, an RNA polymerase III promoter is used to transcribe a short stretch of inverted DNA sequence, which results in the production of a short hairpin RNA (shRNA) that is processed by Dicer to generate siRNAs. These vectors have been widely used to inhibit gene expression in mammalian cell systems.

More recently, several groups have reported the use of RNA polymerase III-based shRNA expression constructs to generate transgenic RNAi mice (6–8), in some cases recapitulating knockout phenotypes (7, 8). Due to the dominant nature of RNAi, a major limitation of this approach is that germ-line transmission can be obtained only for shRNAs targeting genes whose knock-down is compatible with animal viability and fertility. Moreover, even for cell-based applications, constitutive knock-down of gene expression by RNAi can limit the scope of experiments, especially for genes whose inhibition leads to cell lethality.

To overcome these limitations, and to extend the applications of RNAi in mammalian systems, we have developed a Cre-lox-based approach for the conditional expression of shRNA. Two different strategies were used to generate mouse embryonic fibroblasts (MEFs), embryonic stem (ES) cells and transgenic mice in which the expression of an shRNA is tightly regulated in a Cre-dependent manner. One vector allows for conditional activation of shRNA expression, whereas the other permits conditional inactivation of expression of the hairpin RNA. When combined with a variety of Cre expression strategies, these vectors add a powerful capability in the use of RNAi to control mammalian gene expression.

Materials and Methods

Generation of Plasmids. To generate the plasmid for stable RNAi, conditional (pSico), the lox-CMV-GFP-lox cassette was removed from lentilox 3.7 (pLL3.7; ref. 7) by digesting with *Bfu*AI and *Pci*I, followed by filling-in and religation. The first bifunctional lox site (hereafter termed "TATAlox"), followed by the terminator and by an *Eco*RI, was inserted in the resulting plasmid by PCR-mediated mutagenesis using the following oligos: pSico6Eco, GAATTCAACGCGCGGTGACCCTCGAGG; and pSico6, ASAAAAAACCAAGGCTTATAACTTC-GTATAATTTATACTATACGAAGTTATAATTACTTTA-CAGTTACCC.

To insert the second TATAlox preceded by a *Not*I site, the resulting plasmid was digested with *Eco*RI and *Xho*I and ligated to the following annealed oligos: TATALOX F, AATTCGA-GAGGCGGCCGCATAACTTCGTATAGTATAAATTA-TACGAAGTTATAAGCCTTGTTAACGCGCGGTGACCC; and TATALOX R, TCGAGGGTCACCGCGCGTTAACAA-GGCTTATAACTTCGTATAATTTATACTATACGAA-GTTATGCGGCCGCCTCTCG.

The resulting construct was finally digested with *Eco*RI and *Not*I and ligated to an *Eco*RI-CMV-GFP-*Not*I cassette to generate pSico. A similar strategy was used to generate the various "test" constructs shown in Fig. 6, which is published as supporting information on the PNAS web site. Primer sequence and details are available upon request.

To generate pSico Reverse (pSicoR) the 5′ loxP site present in pLL3.7 was removed by digesting with *Xho*I and *Not*I and replaced with a diagnostic *Bam*HI site by using the following annealed oligos: Lox replace for TCGAGTACTAGGATCCAT-TAGGC and Lox replace rev GGCCGCCTAATGGATCCT-AGTAC.

A new lox site was inserted 18 nt upstream of the proximal sequence element (PSE) in the U6 promoter by PCR-mediated mutagenesis.

Abbreviations: shRNA, short hairpin RNA; siRNA, small interfering RNA; RNAi, RNA interference; ES, embryonic stem; Npm, nucleophosmin; Dnmt1, DNA methyltransferase 1; MEF, mouse embryonic fibroblasts; dsRNA, double-stranded RNA; pSico, plasmid for stable RNAi, conditional; pSicoR, pSico reverse; PSE, proximal sequence element; DSE, distal sequence element; TATAlox, a bifunctional lox site; Ad, adenovirus; Ad-Cre, Cre-expressing recombinant Ad.

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Oligos coding for the various shRNAs were annealed and cloned into *Hpa*I–*Xho*I-digested pLL3.7, pSico, and pSicoR. Oligo design was as described (7). The following target regions were chosen: Nucleophosmin (Npm), GGCTGACAAAGACTATCAC; Luciferase, GAGCTGTTTCTGAGGAGCC; DNA methyltransferase 1 (Dnmt1), GAGTGTGTGAGGGAGAAA; and P53, GTACTCTCCTCCCCTCAAT.

The CD8 oligo sequence was the same described in ref. 7. All constructs were verified by DNA sequencing. To amplify recombined and unrecombined vector the following oligos were used: Loopout F, CCCGGTTAATTTGCATATAATATTTC; and Loopout R, CATGATACAAAGGCATTAAAGCAG.

Virus Generation and Infection. Lentiviruses were generated essentially as described (7). Briefly, 5 μg of lentiviral vector and 2.5 μg of each packaging vector were cotransfected in 293T cells by using the FuGENE 6 reagent (Roche Diagnostics). Supernatants were collected 36–48 h after transfection, filtered through a 0.4-μm filter, and used directly to infect MEFs. Two rounds of infection 8 h apart were usually sufficient to infect >90% of cells. GFP-positive cells were sorted 3–4 days after infection. For ES cell infection, the viral supernatant was centrifuged at 25,000 rpm in a Beckman SW41t rotor for 1.5 h, the viral pellet was resuspended in 200 μl of ES cell medium, and was incubated 6 h at 37°C with 10,000–20,000 cells. After infection, ES cells were plated in 10-cm dishes with feeders and GFP-positive colonies were isolated 4–5 days later. On average, 10–30% of ES colonies were GFP-positive.

Recombinant adenoviral stocks were purchased from the Gene Transfer Vector Core facility of University of Iowa College of Medicine (Iowa City, IA). Infections were performed by using 100 plaque-forming units of virus per cell.

ES Cell Manipulation, Generation of Chimeras, and Tetraploid Complementation. V6.5 ES cells were cultivated on irradiated MEFs in DMEM containing 15% FCS, leukemia-inhibiting factor, penicillin/streptomycin, L-glutamine, and nonessential amino acids. MEFs were cultivated in DMEM and 10% FCS supplemented with L-glutamine and penicillin/streptomycin. The derivative of V6.5 containing a doxycycline-inducible Cre transgene in the collagen locus will be described elsewhere (C. Beard and R.J., unpublished data).

B6D2F2 diploid blastocysts and B6D2F2 tetraploid blastocysts were generated and injected with ES cells as described (9). Tetraploid blastocyst-derived animals were delivered by cesarean section on postnatal day 19.5 and fostered to lactating BALB/c mothers. Alternatively, embryonic day 14.5 embryos were surgically removed to generate MEFs following standard procedure. Msx2-Cre mice (10) were received from G. Martin (University of California, San Francisco) and Lck-Cre mice (11) were obtained from The Jackson Laboratory.

Southern Blot and Methylation Analyses. DNA was isolated from the indicated ES cell lines. To assess the levels of DNA methylation, genomic DNA was digested with *Hpa*II and was hybridized to pMR150 as a probe for the minor satellite repeats (12). For the methylation status of imprinted loci, a bisulfite conversion assay was performed by using the CpGenome DNA modification kit (Chemicon), using PCR primers and conditions already described (13). PCR products were gel-purified, digested with *Bst*UI, and resolved on a 2% agarose gel.

Northern Blots. For the small RNA Northern blotting, 15 μg of total RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions, and was resolved on a 15% denaturing polyacrylamide gel, transferred to a nylon membrane, and was cross linked by using the autocrosslink function of a Stratalinker. The membrane was hybridized overnight to a ^{32}P 5′-labeled DNA probe corresponding to the 19-nt sense strand of the p53 shRNA (GTACTCTCCTCCCCTCAAT). Hybridization and washes were performed at 42°C.

For detection of the p53 mRNA, 15 μg of total RNA was resolved on an agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized to a probe corresponding to the entire p53 coding sequence.

Antibodies, Chemicals, and Flow Cytometry. Anti-α-tubulin antibody was from Sigma, the p53 antibody was a kind gift by K. Helin (European Institute of Oncology, Milan), and the anti-Npm was a gift from P. G. Pelicci (European Institute of Oncology, Milan) and E. Colombo (European Institute of Oncology, Milan). All mouse monoclonal antibodies were used. Doxorubicin and doxycycline were obtained from Sigma.

To assess expression of CD4 and CD8 in mice, single-cell suspensions of splenocytes were blocked with anti-CD16/CD32 for 10 min on ice. After blocking, the cells were incubated with phycoerythrin-conjugated anti-CD8, allophycocyanin-conjugated anti-CD4, and PerCPCy5.5-conjugated anti-CD3 for 20 min at 4°C (BD Pharmingen, San Diego). Acquisition of samples was performed on a FACScan flow cytometer, and the data were analyzed with CELLQUEST software (BD Immunocytometry Systems, San Jose, CA). Plots were gated on CD3$^+$ cells

For cell-cycle analysis, 10^6 cells were fixed in 70% ethanol, washed in PBS, and resuspended in 20 μg/ml propidium iodide (Sigma) and 200 μg/ml RNAseA in PBS.

Luciferase Assay. For reporter assay, 293T cells were cotransfected in 12-well plates by using FuGENE 6 with the appropriate shRNA vectors and pGL3control and pRLSV40. The total amount of transfected DNA was 500 ng per well. Firefly and Renilla luciferase activity were measured 36 h after transfection by using the dual reporter kit (Promega) according to the manufacturer's instruction. All experiments were performed in triplicate.

Results

Generation of pSico and pSicoR. The U6 promoter has been widely used to drive the expression of shRNAs and a U6-based lentiviral vector for the generation of transgenic mice has been recently described (7). To control shRNA expression in a Cre-dependent manner, we decided to modify the mouse U6 promoter by inserting a Lox-STOP-Lox cassette. Similar to other RNA polymerase III promoters, the U6 promoter is extremely compact, consisting of a tightly spaced TATA box, a PSE, and a distal sequence element (DSE; Fig. 1*A*). Mutagenesis experiments have demonstrated that while the DSE is partially dispensable for transcriptional activity, the PSE and the TATA box are absolutely required. Moreover, the spacing between the PSE and the TATA box (17 nt) and between the TATA box and the transcription start site (25 nt) is critical, because even small changes have been shown to severely impair promoter activity (14). A consequence is that to effectively suppress the activity of the U6 promoter, the Lox-STOP-Lox element must be positioned either between the PSE and the TATA box or between the TATA box and the transcription start site. In addition, to reconstitute a functional promoter, after Cre expression, the normal spacing between PSE, TATA box, and transcription start site must be restored. The latter consideration precludes the utilization of a classic lox-STOP-lox cassette because, after Cre-mediated recombination, the residual loxP site (34 nt) would necessarily increase the PSE-TATA or the TATA-start-site spacing (See Fig 6).

To overcome these limitations, we generated a bifunctional lox site (TATAlox), that, in addition to retaining the ability to undergo Cre-mediated recombination, contains a functional TATA box in its spacer region (Fig. 1 *B–D*).

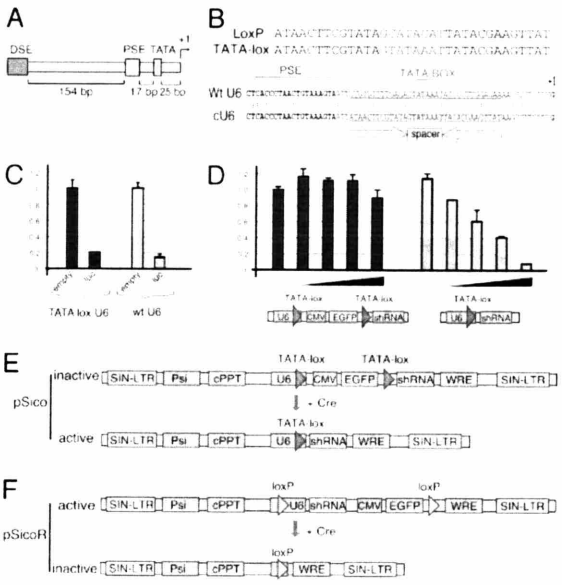

Fig. 1. Generation of pSico and pSicoR. (*A*) Schematic representation of the mouse U6 promoter. The spacing between the DSE, the PSE, the TATA box, and the transcription start site (+1) is indicated. (*B*) Comparison between the sequence of a loxP site and a TATAlox site (*Upper*). Comparison between the sequence of the wild-type mouse U6 promoter and the sequence of the U6 promoter with a TATAlox site replacing the TATA box (*Lower*). (*C*) The TATAlox can replace the TATA box in the U6 promoter. Equal amounts of the wild-type U6 promoter and of the TATAlox U6 promoter (empty or driving the expression of shRNA against the firefly luciferase gene) were transfected in 293T cells together with reporter plasmids expressing firefly luciferase and renilla luciferase. Thirty-six hours later, cells were lysed and the ratio between firefly and renilla luciferase activity was measured. (*D*) A TATAlox-STOP-TATAlox cassette in the U^- promoter efficiently suppresses shRNA expression. Increasing amounts (0–200 ng) of plasmids containing the indicated version of the U6 promoter were transfected in 293T cells together with reporter plasmids, and luciferase activity was measured as in *C*. (*E*) Schematic representation of pSico before and after Cre-mediated recombination. (*F*) Schematic representation of pSicoR before and after Cre-mediated recombination. SIN-LTR, self-inactivating long terminal repeats; Psi, required for viral RNA packaging; cPPT, central polypurine tract; EGFP: enhanced GFP; WRE, woodchuck regulatory element.

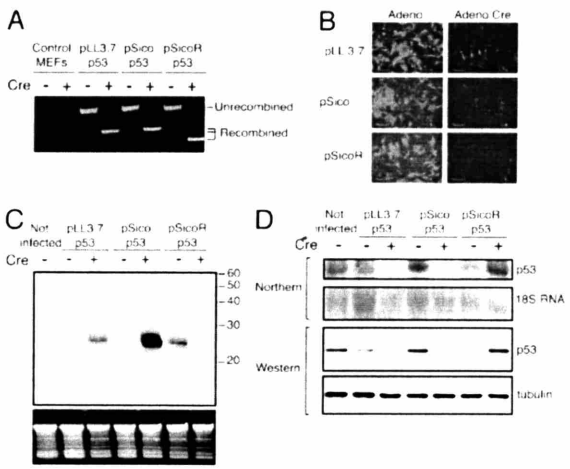

Fig. 2. Cre-regulated knockdown of p53. (*A*) $p53^{R270H/-}$ MEFs infected with the indicated lentiviruses were sorted for GFP positivity and infected with Ad or Ad-Cre. Four days after infection, genomic DNA was extracted, and a PCR was performed to amplify the recombined and unrecombined viral DNA. (*B*) The same cells were analyzed by epifluorescence microscopy to detect GFP. Similar cell density and identical exposure time was used for all images. (*C*) Fifteen micrograms of total RNA extracted from the above indicated MEFs was separated on a 15% denaturing polyacrylamide gel, transferred on a nitrocellulose filter, and hybridized to a radi-labeled 19mer corresponding to the sense strand of the p53 shRNA. Equal RNA loading was assessed by ethidium bromide staining of the upper part of the gel (*Lower*). (*D*) Northern (*Upper*) and Western blotting (*Lower*) showing p53 knock-down in the above indicated cells.

As shown in Fig. 1, when the TATAlox replaces the TATA box site in the U6 promotor, the spacing between PSE, TATA, and transcriptional start site is not altered (Fig. 1*B*), and the resulting promoter retains transcriptional activity (Fig. 1*C*).

To create a conditional U6 promoter, a cytomegalovirus (CMV)-enhanced GFP stop/reporter cassette was inserted between two TATAlox sites so that after Cre-mediated recombination the cassette would be excised, generating a functional U6 promoter with a TATAlox in place of the TATA box (Fig. 1*D*). A T_6 sequence was positioned immediately upstream of the CMV promoter to serve as a termination signal for RNA polymerase III. The terminator combined with the inserted CMV-GFP cassette completely suppressed the activity of the U6 promoter (Figs. 1*D* and 2 *C* and *D*). To facilitate the generation of conditional knock-down mice and cell lines, the conditional U6 cassette was inserted into a self-inactivating lentiviral vector derived from pLL3.7 (7). The resulting plasmid was named pSico (Fig. 1*E*).

To allow for conditional inactivation of shRNA expression, we generated a second vector named pSicoR (Fig. 1*F*). In pSicoR, the CMV-GFP reporter cassette is placed downstream of the U6 promoter and does not affect its activity. Two loxP sites in the same orientation are present in this vector; the first positioned immediately upstream of the PSE in the U6 promoter, and the second immediately downstream of the GFP-coding sequence. In contrast to cells infected with pSico, cells infected with pSicoR are expected to constitutively transcribe the desired shRNA until a Cre-mediated recombination event leads to the excision of the CMV-GFP cassette and an essential part of the U6 promoter. Importantly, in both pSico and pSicoR, the CMV-GFP cassette marks infected cells and loss of GFP expression indicates successful Cre-mediated recombination.

Cre-Regulated RNAi in Cells. The ability of pSico and pSicoR vectors to conditional silence endogenous genes was demonstrated by insertion of a hairpin designed to inhibit expression of the mouse tumor suppressor gene p53. As a control, the same sequence was cloned into the constitutive shRNA vector pLL3.7. In pLL3.7, the CMV-GFP cassette is located downstream of the U6 promoter and is flanked by loxP sites such that Cre-mediated recombination is expected to result in loss of GFP expression without affecting shRNA expression (7). These three constructs were then used to generate lentiviruses and infect MEFs. To simplify the detection of p53, MEFs expressing high basal levels of a transcriptionally inactive point mutant (R270H) p53 allele (K. Olive and T.J., unpublished work) were used in these experiments. High-efficiency transduction by all of these vectors was achieved as indicated by uniform GFP expression in infected cells (Fig. 2*B* and data not shown). As shown in Fig. 2, after superinfection with a Cre-expressing recombinant adenovirus (Ad-Cre), near complete recombination with concomitant loss of GFP fluorescence was observed for all vectors. One week after Cre expression, high levels of the p53-siRNA were detected in cells infected with pSico-p53 (Fig. 2*C*), whereas no p53-siRNA

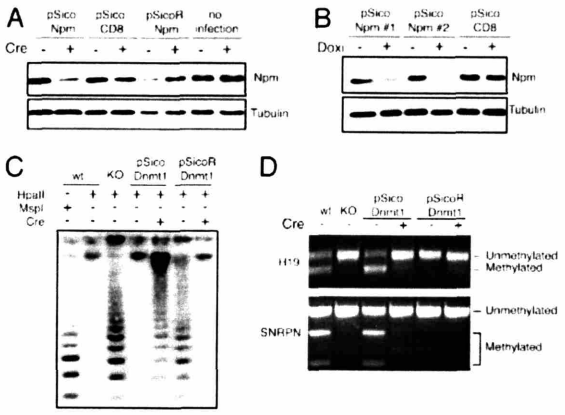

Fig. 3. Cre-regulated knockdown of Npm and Dnmt1. (*A*) Cre-regulated knock-down of Npm. MEFs were infected with the indicated lentiviruses, and GFP-positive cells were sorted and were superinfected with empty Ad or Ad-Cre. One week later, whole-cell lysates were separated by SDS/PAGE, and were subjected to Western blotting against Npm and tubulin. (*B*) ES cells carrying a doxycycline-inducible Cre (C. Beard and R.J., unpublished data) were infected with the indicated lentiviruses. GFP-positive clones were isolated, passaged two times, and were either left untreated or were incubated with 2 μg/ml doxycycline for 1 week. Immunoblot analysis was performed as in *A*. (*C*) Cre-regulated knock-down of Dnmt1 affects cytosine methylation. Methylation analysis of minor satellite DNA. ES cells carrying a doxycycline-inducible Cre transgene were infected with the indicated lentiviruses. Single GFP-positive clones were isolated, expanded, and passaged five times before being either mock-treated or incubated with 2 μg/ml doxycycline. After five more passages, the genomic DNA was extracted and digested with the indicated enzymes and subjected to Southern blot analysis. (*D*) As in *C*, but the genomic DNA was treated with sodium bisulfite, subjected to PCR to amplify the indicated imprinted regions, and digested with *Bst*UI.

was observed in the same cells in the absence of Cre expression, confirming the complete suppression of U6 promoter activity by the TATAlox-STOP-TATAlox cassette. The length of the processed RNA (21–24 nt) was identical in cells infected with pLL3.7-p53, pSico-p53 (after Ad-Cre infection), or pSicoR-p53 (before Ad-Cre infection), indicating that the presence of the TATAlox in pSico did not qualitatively affect siRNA production. Finally, infection with Ad-Cre led to almost complete disappearance of p53-siRNA in pSicoR-p53-infected cells (Fig. 2*C*).

Consistent with functional p53-siRNA expression by these vectors, Cre-mediated recombination resulted in a dramatic reduction of both p53 mRNA and protein levels in pSico-p53-infected cells (Fig. 2*D*). Conversely, pSicoR-p53 generated a p53 knock-down that was reversed upon Ad-Cre infection (Fig. 2*D*). We noticed an unexpected increase in p53-siRNA and p53 knock-down after Cre expression in cells infected with pLL3.7-p53 (Fig. 2 *C* and *D*, lanes 2 and 3). This increase could reflect promoter interference because the CMV and the U6 promoters are in close proximity in pLL3.7 before Cre-mediated recombination.

As additional proof of concept, we cloned short hairpins directed against the nucleolar protein Npm and the DNA methyl transfrase Dnmt1 into pSico and pSicoR. Npm is a putative tumor-suppressor gene involved in a number of chromosomal translocations associated with human leukemias and lymphomas, and has been shown to physically and functionally interact with the tumor suppressors p19ARF and p53 (15, 16). Specific, Cre-dependent knock-down of Npm was observed in both MEFs and ES cell clones infected with pSico-Npm (Fig. 3 *A* and *B*). The opposite effect, Cre-dependent reexpression of Npm, was observed in pSicoR-Npm-infected MEFs (Fig. 3*A*, and Fig. 7, which is published as supporting information on the PNAS web site).

The characterization of ES cells mutant for Dnmt1 has been reported (17), and demonstrated that Dnmt1 is required for genome-wide maintenance of cytosine methylation. Dnmt1-deficient ES cells are viable and proliferate normally, despite substantial loss of cytosine methylation; however, they die upon differentiation. Whereas reexpression of the Dnmt1 cDNA in these cells leads to methylation of bulk genomic DNA and nonimprinted genes, the methylation pattern of imprinted loci cannot be restored without germ-line passage (18, 19). We tested whether we could recapitulate the phenotype observed in Dnmt1-deficient ES cells by using pSico-Dnmt1 and pSicoR-Dnmt1. As shown in Fig. 3, pSico-Dnmt1-infected ES cells underwent significant loss of CpG methylation of minor satellites (Fig. 3*C*) and of two imprinted genes tested (Fig. 3*D*) upon Cre induction. Importantly, the reacquisition of DNA methylation at minor satellite sequences, but not at imprinted loci in pSicoR-Dnmt1 after Cre-mediated recombination, confirms previous results obtained with reexpression of Dnmt1 (19). These results further illustrate the potential for application of the pSicoR vector *in vitro* and *in vivo* to perform "rescue" experiments.

Conditional RNAi in Mice. One motivation for incorporating a conditional U6 cassette into a lentiviral vector was to rapidly generate conditional knock-down mice. To demonstrate this application directly, ES cells were infected with pSico-CD8 (Fig. 4*A*), which was designed to inhibit expression of the T lymphocyte cell surface marker CD8 (7). Three pSico-CD8 ES clones were used to generate chimeric mice, and transmission of the pSico-CD8 transgene to the progeny was observed for two of them. All transgenic mice were easily identified by macroscopic GFP visualization (Fig. 4*B*), although we observed some variability in the extent and distribution of GFP expression among littermates. Importantly, all transgenic mice produced normal amounts of $CD4^+$ and $CD8^+$ lymphocytes and were apparently normal and fertile, indicating that the presence of the nonexpressing pSico-CD8 transgene before Cre activation did not affect CD8 expression and was compatible with normal mouse development.

To achieve either global or tissue-specific activation of the CD8 shRNA, pSico-CD8 chimeras were crossed to Msx2-Cre or Lck-Cre transgenic mice that express Cre in the oocyte (10, 20), or under the control of a T cell-specific promoter (11), respectively. Fluorescence-activated cell sorter analysis demonstrated that pSico-CD8;Lck-Cre and pSico-CD8;Msx2-Cre mice had a specific reduction in splenic $CD8^+$, but not $CD4^+$ T lymphocytes as compared with controls (Fig. 4*C*). As predicted, the pSico-CD8;Msx2-Cre progeny showed complete recombination of the pSicoCD8 transgene and lacked detectable GFP expression, although in the pSico-CD8;Lck-Cre mice recombination was detected in the thymus but not in other tissues (Fig. 4*D* and data not shown). Transgenic mice derived from two different ES clones gave similar results.

Tetraploid blastocyst complementation represents a faster alternative to diploid blastocyst injection because it allows the generation of entirely ES-derived mice without passage through chimeras (9, 21). In principle, this technology applied to pSico-infected ES cells would allow the generation of conditional knock-down mice in ≈5–6 weeks (1 week for cloning the shRNA, 1–2 weeks for ES cell infection and clone selection, and ≈2 weeks for tetraploid blastocyst injection and gestation). To test this protocol directly, ES cells were infected with pSico-p53 and two different clones, pSico-p53#1 and pSico-p53#3, were injected into tetraploid blastocysts. As a rapid way to assess the inducibility of the p53 shRNA in ES cell-derived animals, midgestation embryos were recovered from two recipient females. Two apparently normal, GFP-positive embryos were recovered; one

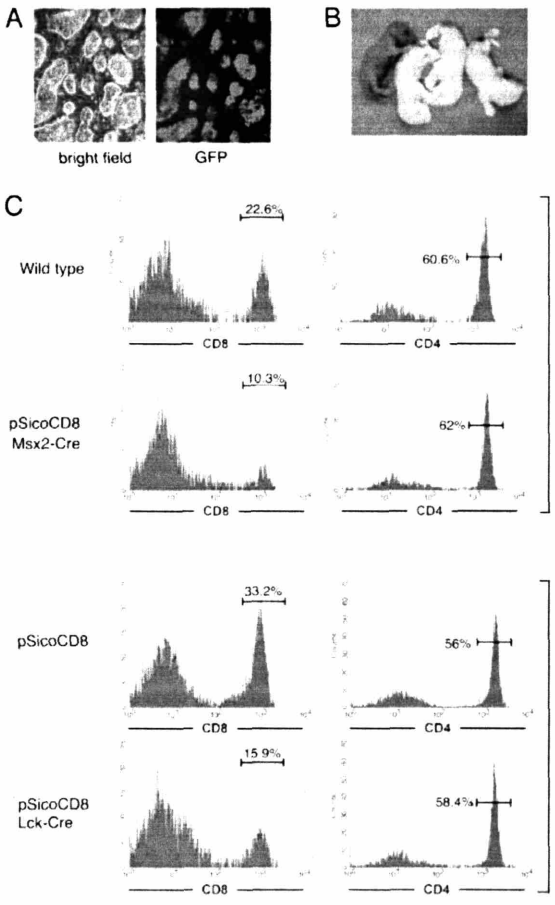

Fig. 4. Conditional knockdown of CD8 in transgenic mice. (*A*) ES cells infected with pSico-CD8 visualized with an inverted fluorescence microscope. (*B*) A litter of newborns derived from a cross between a pSico-CD8 chimera and an Lck-Cre female. Three pups present bright GFP fluorescence, indicating germ-line transmission of the pSico-CD8 transgene. (*C*) Knock-down of CD8 in the spleen of Msx2-Cre × pSico-CD8 and Lck-Cre × pSico-CD8 mice. Chimeras from pSico-CD8-infected ES cells were crossed to Msx2-Cre or Lck-Cre animals. The resulting mice were genotyped for the presence of Cre and pSico. Splenocytes from 1- to 3-week old mice with the indicated genotypes were harvested, stained for CD3, CD4, and CD8 expression, and analyzed by flow cytometry. Only $CD3^+$ cells were plotted. One representative example of littermates for each cross is shown.(*D*) PCR detection of Cre-mediated recombination of pSico-CD8 in genomic DNA extracted from the tail (*A*) or the thymus (*B*) of mice with the indicated genotypes.

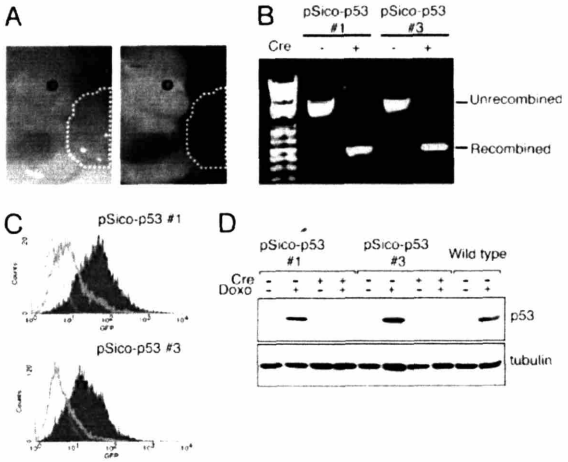

Fig. 5. Generation of conditional knockdown embryos by tetraploid complementation. (*A*) A postnatal day 14.5 embryo derived by tetraploid complementation using the pSico-p53 #1 ES clone. The area enclosed by the dashed line corresponds to the non-ES cell-derived placenta. (*B*) PCR detection of recombination in MEFs derived from the indicated embryos. Genomic DNA was extracted 4 days after Ad or Ad-Cre infection and subjected to PCR. (*C*) Histogram overlays showing loss of GFP expression in MEFs derived from pSico-p53#1 (*Upper*) and pSico-p53#3 (*Lower*) embryos 4 days after Ad-Cre (green plot) or Ad empty (purple filled plot) infection. Control, GFP-negative MEFs (red plot) are included as reference. (*D*) MEFs derved from the indicated tetraploid complementation pSico-p53 embryos, or from wild-type embryos, were treated with doxorubicin for 18 h and subjected to Western blot against p53 and β-tubulin.

each from ES clone pSico-p53 #1 and pSico-p53 #3 (Fig. 5*A* and data not shown). MEFs generated from these embryos were passaged once and infected with Ad or Ad-Cre. As expected, Cre expression induced significant recombination and loss of GFP expression (Fig. 5 *B* and *C*). Importantly, in Ad-Cre-infected cells, p53 induction and cell-cycle arrest after doxorubicin treatment were significantly inhibited compared with Ad-infected control cells (Fig. 5*D*, and Fig. 8, which is published as supporting information on the PNAS web site).

Discussion

Here, we describe two lentivirus-based vectors for conditional, Cre-lox-regulated RNAi in cells and mice; one for Cre-dependent activation (pSico) and one for Cre-dependent termination (pSicoR) of shRNA expression. These vectors were used to demonstrate conditional and reversible knock-down of p53, Npm, and Dnmt1 in ES cells and MEFs. As a proof of principle, pSico was used to generate conditional and tissue-specific knock-down mice.

Since the development of gene targeting technologies in ES cells (22), the gold standard for the analysis of gene function in mammals has been the creation of knock-out mice. Improvements to this technology have allowed refined analysis of gene function at specific developmental stages or in specific tissues, based on conditional knock-out strategies by means of Cre-lox-regulated recombination (23). Despite significant improvements over the last decade, however, the creation of loss-of-function alleles in the mouse remains time consuming and costly. The recent demonstration that constitutive expression of shRNAs driven by RNA polymerase III promoters can be used to functionally silence gene expression in transgenic mice suggests that RNAi-based technologies might represent a convenient alternative to gene targeting through homologous recombination (6–8).

A major limitation of current approaches for transgenic RNAi is that they do not allow regulated expression of shRNA, but

instead cause constitutive gene silencing in all tissues. The two lentiviral vectors described here overcome this limitation.

The compact nature of RNA polymerase III promoters (14) prevents the use of a conventional Lox-STOP-lox strategy to achieve Cre-inducible shRNA expression. Other investigators have recently addressed this problem by placing the lox-STOP-lox cassette in the loop region of the shRNA (24, 25). However, by using this approach, after Cre-mediated recombination, the residual loxP site is transcribed within the shRNA, resulting in the synthesis of a longer dsRNA that is significantly less efficiently processed (24) and could be more prone to elicit non-specific, off-target effects or an IFN response (3). By using a mutant lox site containing a functional TATA box in its spacer sequence, we were able to obtain Cre-regulated transcription and efficient processing of a normal-length shRNA.

We further extend the potential applications of RNAi-based technologies by describing a lentiviral vector (pSicoR) in which constitutive shRNA expression can be terminated by a Cre-mediated recombination event. As we demonstrate for Dnmt1, this vector can be used to determine the functional consequences of gene reactivation and will facilitate rescue experiments *in vivo*. In addition, by mimicking the action of small-molecule drugs designed to activate the proteins or pathways controlled by human disease genes (e.g., tumor suppressor gene), this strategy could be applied to identify promising novel targets for drug development.

Because preparation of conditional RNAi constructs requires merely cloning of short synthetic DNA sequences, a large number of conditional knock-down strains can be generated in parallel by a single investigator. This approach is thus ideally suited for large-scale projects aimed at the characterization of genetic pathways or at the validation of candidate target genes identified through gene profiling screenings. For example, gene expression profiling by using mouse cancer models typically yields numerous genes that distinguish tumor from normal tissue. By using conventional or conditional knockout strategies, it is practical to examine only a small fraction of these genes for functional relevance to tumorigenesis. In contrast, shRNA-conditional systems such as pSico can greatly reduce the time, cost, and effort required to perform experiments of this magnitude.

We note that although in this work pSico and pSicoR were used to control the expression of "artificial" shRNAs, they might also be used to achieve spatially and temporally regulated expression of naturally occurring microRNAs, an approach that could help unravel the biological functions of this abundant class of small RNAs (26).

In summary, the lentiviral vectors reported here represent a significant improvement over constitutive shRNA expression systems and expand the number of potential applications of RNAi-based technologies.

Note. While this manuscript was in preparation, a similar strategy for lentivirus-mediated, Cre-dependent shRNA expression in cells was reported (27). Although our vector and the vector described by Tiscornia *et al.* (27) are both based on a STOP cassette flanked by TATA box-containing lox sites, the presence of the GFP reporter in pSico offers the advantage of marking infected cells and permits the visual detection of successful recombination. Importantly, we show that this strategy can be used to achieve tissue-specific, conditional RNAi in transgenic mice. The demonstration that pSico undergoes efficient tissue-specific recombination both *in vitro* and *in vivo* is of particular relevance because a lox site containing a double mutation in the spacer region identical to the one present in the TATAlox had been previously shown to undergo less efficient recombination compared with a wild-type LoxP site (28).

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Appendix E

"Caspase shRNA sequences."

Christopher Dillon

Summary of all caspase shRNA designed and tested in this project. + indicates >50%, - indicates <50% silencing, ND indicates that silencing was not tested with given method.

			Validated by		
Caspase		Sequence	Western	RT-PCR	Luciferase
1	A	GCCAAATCTTTATCACTTA	+	ND	+
	B	GAAGGCCCATATAGAGAAA	+	ND	+
2	A	GCCGTCCTTTGTAGGATGT	+	ND	+
	B	GACTTACTGCTCACAACCC	+	ND	+
	D	GATGAGACTGCCTACTCGC	-	ND	+
3	1	GCTGTCAGGGAGACTCTCA	+	+	ND
	2	GCAAGTAACTGTCAATGAT	+	ND	ND
	3	GGAGCAAGTAACTGTCAAT	+	ND	ND
6	B	GTGAAATGCTTTAACGACC	-	ND	ND
	C	GGAGACAAGTGTCAGAGCC	-	ND	ND
	E	GTAGGGCCATCTGTCTTGC	-	ND	ND
	F	GGAAATTACTATGCATCAA	+	ND	-
	G	GCTGCATTTCTGTCCCAAA	-	ND	-
7	1	GGTTGATTTCCTGTTTCTG	+	ND	+
	2	GCAGTTTCTTTCCAAGGTA	+	ND	ND
8	A	GAGGCTCTGAGTAAGACCT	-	ND	+
	B	GACCTTTAAGGAGCTTCAT	-	ND	ND
	C	GCAGATGCACAGCCCACC	-	ND	ND
	E	GAAGTGAGCGAGTTGGAAT	-	ND	-
	21	GCACAGAGAGAAGAATGAGCC	-	ND	-
	K	GAACTGGGCAGTGAAGACC	-	ND	-
	N	GAAGCTCTTCTTCCCTCCC	-	ND	-
	F1	GATCGAGGATTATGAAAGA	ND	ND	+
	F2	GCAACAGAACCACACTTTA	ND	ND	+
	F3	GGAAGATCGAGGATTATGA	ND	ND	+
	F4	GGACTTCAGACAAAGTTTA	+	ND	+
	F5	GAATGGCTACGGTGAAGAA	ND	ND	+
	F6	GGAAAGATGTCCTCAAGGA	ND	ND	+
	F7	GTGGATGAGTCTAATTTAT	ND	ND	+
	F8	GTACACACACATACATACA	+	ND	+
9	1	GCTCTTCTTCATCCAGGCC	-	ND	ND
	A	GGCACCCTGGCTTCACTC	-	ND	ND
	B	GAGCTCTGGCCTGCTCGGC	-	ND	ND
	D	GGCCCTTGGACCAGCTGGA	-	ND	ND
	E	GACCTGCAGTCCCTCCTTC	+	ND	ND
11	A	GGAGGCCAATGGCCGTACA	ND	ND	ND
	B	GCAATGTACTGAAATTAAA	ND	ND	ND
12	A	GCATGCCTTGAAATTGTGA	ND	ND	ND
	B	GGAAGATCTTATCTACAAT	ND	ND	ND

Appendix F

"RNAi as an experimental and therapeutic tool to study and regulate physiological and disease processes."

Dillon CP, Sandy P, Nencioni A, Kissler S, Rubinson DA, Van Parijs L.

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RNAi AS AN EXPERIMENTAL AND THERAPEUTIC TOOL TO STUDY AND REGULATE PHYSIOLOGICAL AND DISEASE PROCESSES

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Key Words microRNA, siRNA, shRNA, vector, therapy

■ **Abstract** Over the past four years RNA interference (RNAi) has exploded onto the research scene as a new approach to manipulate gene expression in mammalian systems. More recently, RNAi has garnered much interest as a potential therapeutic strategy. In this review, we briefly summarize the current understanding of RNAi biology and examine how RNAi has been used to study the genetic basis of physiological and disease processes in mammalian systems. We also explore some of the new developments in the use of RNAi for disease therapy and highlight the key challenges that currently limit its application in the laboratory, as well as in the clinical setting.

INTRODUCTION

Mammalian tissue culture and animal models are indispensable tools to study the genetic basis of human physiology and disease. Systematic manipulation of the genetic background by overexpression, deletion, or mutation of genes is the principal method for understanding complex biological processes. Indeed, transgenic, knockout and knockin mice are often the best available in vivo models for human disorders. However, generating these genetically engineered animals requires a significant amount of time, money, and effort. Furthermore, creating a more complex genetic environment with simultaneous gain- and loss-of-function mutations of multiple genes, as is often seen in human diseases, is frequently beyond the reach of these technologies and model systems.

The discovery that long double-stranded RNA molecules (dsRNA) can induce sequence-specific silencing of gene expression in primitive organisms, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, revealed a previously unknown mechanism of gene regulation that is highly conserved throughout

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multicellular organisms (1). This process is called RNA interference (RNAi), and is also known as posttranscriptional gene silencing (PTGS) in plants (2). Initial efforts to apply RNAi in mammalian cells were hindered by a potent cellular response to long dsRNAs initiated by the dsRNA-dependent protein kinase (PKR) (3, 4). This stress response has evolved as a defense against viruses and functions to block viral reproduction by halting protein translation and triggering apoptosis of infected cells (5). The breakthrough discovery that dsRNAs of 21 nucleotides in length (termed small interfering RNAs; siRNAs) could trigger sequence-specific gene silencing without inducing the PKR response (6) has opened up revolutionary new approaches to manipulate gene function in mammalian systems.

In the laboratory, RNAi has proven to be a simple, cheap, and powerful tool to generate cells, tissues, or even animals with reduced expression of specific genes. RNAi has been used to interrogate the function of candidate genes and, more recently, following the creation of random and directed siRNA libraries, has permitted phenotype-driven, forward genetic analysis of normal physiological and disease processes. The development of stable and inducible expression vectors driving the expression of short hairpin RNAs (shRNAs) has further expanded the application of RNAi both in tissue culture and in animal models. Furthermore, a number of successful animal trials indicate that RNAi might ultimately become a potent therapeutic approach for the treatment of various human diseases. While the groundwork for using RNAi to manipulate gene expression in animal systems has been laid down, lingering questions about the specificity of the technique and the possibility of inducing adverse cellular responses remain. Future work in this rapidly evolving field will likely address these issues and extend the applications of RNAi both in the laboratory and in the clinic.

THE BIOLOGY OF RNAi

RNAi as a Novel Mechanism that Regulates Development, Normal Physiology, and Disease

The initial description of RNAi resulted from the finding that the introduction of exogenous long dsRNAs in *C. elegans* caused sequence-specific loss of expression of mRNAs (7). Since then RNAi has been observed in most eukaryotes, with the notable exception of *Saccharomyces cerevisiae* (8). The evolutionary conservation of this process is thought to reflect the importance of a class of short noncoding RNAs, termed microRNAs (miRNAs). These RNAs were initially discovered in a screen for genes required for larval development in *C. elegans* (9). More recently, several groups have identified hundreds of miRNAs in species ranging from *C. elegans* to humans through experimental and computational strategies (10–13). A recent study has shown that overexpression of miRNAs alters the development of immune cells in mice (14), indicating that miRNAs are also likely to be critical for normal development and tissue physiology in mammals. Intriguingly, misexpression of miRNAs has been reported in a number of cancers, suggesting that miRNAs may contribute to disease processes as well (15, 16). To date, little is

known about the targets of most miRNAs (17). In large part this is because these RNAs show imperfect homology with the mRNAs that they regulate.

Overview of the Biochemistry of RNAi

Significant strides have been made in our understanding of the biochemical mechanisms by which endogenous miRNAs silence gene function. Initially, miRNAs are transcribed as single-stranded precursors up to 2 kb in length that exhibit significant secondary structure owing to the presence of stretches of bases that can undergo extensive base pairing followed by stretches that adopt loop structures (18, 19). Importantly, the pairing regions, or stems, present in miRNAs often contain a small number of mismatched bases that create "bubbles" in the miRNA structure (10–12). Together with loop structures, these are important for the recognition of miRNAs by cellular enzymes and for the ability of these RNAs to silence genes.

Primary miRNA transcripts are identified and processed in the nucleus by the RNase III enzyme, Drosha, into approximately 70-nt-long precursors, known as pre-miRNAs (20). These are exported to the cytoplasm, where they are cleaved by a second RNAse III enzyme, Dicer. Dicer converts miRNAs into double-stranded 21- to 23-nt-long mature miRNAs (21). Mature miRNAs associate with an enzymatic machine known as the RNA-induced silencing complex (RISC) (22). The composition of the RISC is not completely defined but includes Argonaute family proteins (23–28). The RISC unwinds miRNAs and associates stably with the (antisense) strand that is complementary to target mRNA (29). The complete enzymatic machinery required to process miRNAs appears to be expressed in most eukaryotic cells and is essential because mice and zebrafish that lack *dicer1* fail to complete development (30, 31).

Depending on the degree of homology between a miRNA and its target mRNA, miRNA-RISC complexes inhibit gene function by two distinct pathways (17). Most miRNAs pair imperfectly with their targets and silence gene expression by translational repression (32–34). This RNAi mechanism appears to operate most efficiently when multiple miRNA-binding sites are present in the 3′UTR of the target mRNAs (35, 36). In some cases, miRNAs exhibit perfect sequence identity with the target mRNA and inhibit gene function by triggering mRNA degradation (22). As discussed below, this appears to be the dominant mechanism by which synthetic siRNAs and plasmid-expressed shRNAs silence gene expression.

Co-Opting the Endogenous RNAi Machinery for Experimental and Therapeutic Purposes

Much of the success of RNAi as a research and potential therapeutic tool is due to the fact that the enzymatic machinery required to process miRNAs is ubiquitously expressed and can be co-opted by exogenous RNAs to direct sequence-specific gene silencing. In plants and primitive eukaryotic organisms, the sequential activity of Drosha and Dicer converts viral, transgene-encoded, and synthetic dsRNAs into siRNAs that trigger gene silencing (37). To avoid stress responses to long dsR-

NAs, gene silencing in mammalian cells is typically induced experimentally by small exogenous RNAs that enter the RNAi pathway further downstream. Synthetic siRNAs are designed to mimic Dicer products so that they can directly associate with the RISC and target homologous mRNAs for degradation (6). Plasmid-expressed shRNAs are thought to enter the RNAi pathway because they are recognized and cleaved by Dicer into products that bind the RISC (38–40). A recent study suggests that it may even be possible to trigger RNAi in mammalian cells by providing a long dsRNA substrate for Drosha, as long as the dsRNA is confined to the nucleus by removing its 5′ cap structure (41).

RNAi AS AN EXPERIMENTAL TOOL

Discovery and Design of siRNAs for Gene Silencing in Mammalian Systems

Pioneering work by Tuschl and colleagues demonstrated that chemically synthesized short 21-mer dsRNAs (siRNAs) were able to silence genes in a sequence-specific manner when introduced into mammalian cells (6). This exciting observation was quickly reproduced by other groups, and subsequent work rapidly defined the basic structure of siRNAs that were able to induce gene silencing, including the need for a 19-nt RNA duplex with 2-nt overhang on the 3′ ends. Most groups substituted (2′-deoxy)thymidine nucleotides for the overhangs, which did not affect silencing but reduced the cost of the oligonucleotides.

As the use of siRNAs to silence gene expression became more widespread, it became apparent that not all sequences within an mRNA could act as targets for RNAi and that therefore a set of rules was necessary to optimize siRNAs. This contrasts to the situation in primitive organisms in which expression of dsRNAs of 500 base pairs or more typically results in very efficient gene silencing, irrespective of the sequence of the target mRNA (7). Initially the selection of siRNA sequences was largely determined using a limited set of empirical guidelines (6, 42). These included factors such as the GC-content and the region of the mRNA targeted (6, 42). On the basis of these guidelines, most investigators were able to achieve efficient gene silencing with about one quarter of all siRNAs.

An important recent development has been the definition of more effective rational rules for siRNA design. The first breakthrough came from analysis of the biochemistry of RNAi and, in particular, the mechanisms by which siRNAs associate with the RISC complex. These studies revealed that only the antisense strand of the siRNA is incorporated into this enzymatic machinery and that miRNAs and effective siRNAs exhibited decreased stability of the 5′ end of the antisense strand (43, 44). The relevance of this finding to enhancing siRNA efficiency was further underscored by demonstrating that base pair mismatches introduced at the 5′ end of siRNAs improved gene silencing (43, 44). A further step was taken by investigators at Dharmacon, who evaluated a number of characteristics of 180 siRNAs directed against two genes (45). Based on this systematic analysis, a

more comprehensive set of criteria was established that significantly increases the likelihood of identifying functional siRNAs (45). It should be noted, however, that highly effective siRNA sequences can be found that do not adhere to these criteria and that some siRNAs that adhere to the criteria do not function well. Therefore, it is likely that future work will continue to define the structure of effective siRNAs.

Stable Induction of RNAi in Mammalian Cells Through Expression of shRNAs

In some organisms, notably *C. elegans*, RNA-dependent RNA polymerases exist that are able to amplify siRNAs and even pass them on through the germ line (46, 47). As a consequence, introduction of dsRNA triggers long-lived, stable gene silencing in this organism (46). These polymerases do not exist in mammalian cells, and consequently, gene silencing induced by siRNAs is limited by the number of RNA molecules introduced into a cell. The number of siRNAs decreases with time by dilution as cells divide and probably also as a consequence of degradation by cellular enzymes. Accordingly, in many cell culture systems, gene silencing is seen for only a few days after siRNAs are administered. On the other hand, siRNA-induced gene silencing has been observed for weeks in slowly proliferating or non-dividing cell types such as macrophages and hepatocytes (48, 49).

The solution to obtaining more universal stable gene silencing through RNAi has been to develop expression systems that stably produce siRNAs. This was first accomplished by several groups (38–40) who used the promoter of either the U6 or H1 splice factor to express a short hairpin RNA (shRNA) that can be processed by Dicer to produce siRNAs. The shRNAs had duplex stems that varied in length from 19 to 29 nt connected by a short loop sequence. The sequence of the loop was critical for effective target silencing; however, several different sequences were shown to be functional. The U6 and H1 promoters recruit RNA polymerase III, a specialized polymerase that is responsible for generating most of the cell's small RNAs (e.g., splicing RNAs and tRNAs) (50). The advantages of using these promoters to create shRNAs are that their transcription initiation site and termination site are well defined and highly conserved and that these promoters are highly active in most, if not all, mammalian cells (50). Importantly, pol III promoter shRNA expression cassettes have been found to be very flexible and have been introduced in a number of different expression systems, including virus-based and those used to create transgenic animals. Several pol III promoters have been extensively characterized and co-opted to express shRNAs. Whether a particular promoter functions significantly better than others in a particular system remains controversial (51–53), and evidence exists that inclusion of an enhancer element may improve their activity (54).

In contrast to promoters for small RNAs, promoters for most cellular genes recruit RNA polymerase II and usually generate transcripts that initiate in a less conserved manner and require a long termination poly (A) sequence. With a few exceptions (41, 55), both features have largely precluded the use of pol II

promoters to generate shRNAs. This is not the case for endogenous miRNAs, which are expressed from pol II promoters in a developmentally regulated or tissue-specific manner (18, 19). Furthermore, miRNAs and, possibly, also slightly longer shRNAs may enter the RNAi pathway more efficiently than typical 19-mer shRNAs, potentially leading to more potent gene silencing (14, 40, 56). For these reasons, new stable expression systems for siRNAs that are based on miRNA structures have been developed by a number of groups (14, 56). Expression of a long nuclear-restricted dsRNA in cells and mice has also been shown to silence gene expression effectively (41), suggesting that further improvements and modifications will be made to stable siRNA delivery systems as our understanding of the RNAi machinery improves.

Regulated and Tissue-Specific Gene Silencing by RNAi

Whereas constitutive expression of siRNAs in cells, tissues, and animals has provided important insights into biological processes, the ability to control gene silencing more tightly is likely to significantly extend the applications of experimental RNAi. For this reason, a significant focus has been on the creation of regulated and tissue-specific siRNA delivery systems. To date, most of these systems are based on engineered pol III promoters that are controlled by small molecules or the Cre recombinase. A number of groups have demonstrated that gene silencing can be initiated or inhibited by Cre-driven recombination of modified pol III promoters in cells and in mice and by introduction of DNA elements that bind tetracycline- or ecdysone-regulated transcriptional activators or repressors (see Table 1). Further refinements are likely to result in more effective and flexible RNAi systems to interfere with gene function in mammals. An alternative approach to obtain regulated expression of siRNAs might be to use pol II promoters. With the exception

TABLE 1 In vivo gene silencing in mammals

Silencing	Method	Reference
siRNA or shRNA	Hydrodynamic shock	(48, 69, 70, 146)
	Cationic liposomes/ complexes	(71, 72, 81, 150)
	Peptide conjugation	(82)
	Electroporation	(73–76)
	Adenovirus	(92–94)
Stable	Retrovirus	(58, 86)
	Lentivirus	(84, 85, 87–90)
	Adeno-associated virus	(55, 91, 98, 99)
	Transgenic	(100–102, 104)
Inducible	Tetracycline	(162–167)
	Cre	(168–171)
	Ecdysone	(172)

of the constitutive cytomegalovirus (CMV) promoter (55), this approach has not yet been shown to be successful, although it is likely that by using miRNA-based structures to deliver siRNAs that these promoters will be used to trigger RNAi in a regulated manner in the future.

Off-Target Effects and Interferon Responses: Possible Limitations to the Use of RNAi

A key feature of siRNAs is that they inhibit genes in a highly specific manner. Indeed, the initial description of these RNAs demonstrated that alteration of a single base pair was sufficient to disrupt gene silencing (6). For many subsequent studies with siRNAs, this specificity was shown to hold true (57, 58). A number of more recent studies have suggested that there are situations where mismatches between the siRNA and target sequence can be tolerated (59). This observation has raised the concern that siRNAs may have effects on genes that are not considered targets, so-called off-target effects. This concern has been addressed by a number of groups that have examined genome-wide changes in gene expression following the introduction of siRNAs. Some of these studies found that a number of genes unrelated to the target are changed in expression, mostly by a factor of twofold. These off-target effects have been correlated with the concentration of siRNAs (60), as well as similarities between the off-target transcripts and the 5′ ends of siRNAs (61, 62). It seems plausible on the basis of recent work that the decreased off-target mRNA levels are the consequence of siRNAs adopting miRNA-like properties, resulting in slightly decreased levels of mRNAs, possibly through alterations in mRNA stability (61). It remains to be determined whether translational inhibition is seen on these off-target mRNAs.

Currently it is not possible to predict whether a particular mammalian siRNA will induce off-target effects. It is widely assumed that the most informative parameter will be the degree of homology between siRNAs and other gene products, and therefore most experimental siRNAs are designed to have no known perfect matches with mRNAs other than the intended target (62a). It is interesting that off-target effects are not observed when dsRNAs are used in primitive organisms. This may be because the dominant species of siRNAs generated from these dsRNAs are selected by Drosha and Dicer and other components of the endogenous RNAi machinery, which might have a proofreading activity that guards endogenous genes from silencing. Thus it is possible that mammalian siRNAs' generated from dsRNAs' precursors through the action of Drosha and Dicer may be less prone to induce off-target effects.

A second major concern among researchers using RNAi in mammals is the possibility that introducing exogenous dsRNAs may trigger an antiviral interferon response mediated by the PKR. Indeed, many early attempts at silencing gene expression using dsRNAs strategies analogous to those developed for primitive organisms failed because they triggered the production of interferon, nonspecific gene silencing, and apoptosis in mammalian cells (63). Early work by Tuschl and colleagues suggested that dsRNAs that were less than 30 bases in length were

able to silence gene expression in a specific manner, while eluding the molecular machinery responsible for triggering the interferon response (6). This finding has been corroborated by the successful use of siRNAs as reagents to interfere specifically with gene function in a wider variety of different mammalian systems. However, a number of recent studies suggest that even short dsRNAs can trigger the expression of some of the target genes of the interferon response and, in some cases, can induce the cellular changes associated with this process (64–66). As of now, it is not clear how often siRNAs and shRNAs trigger the interferon pathway and which conditions favor this response to these RNAs. Chemical features of dsRNAs, as well as their expression levels and delivery routes, may determine whether they become visible to the interferon response machinery (64–66).

Similar to the interferon response, evidence exists that siRNAs and shRNAs can activate dendritic cells and other cells of the immune system through a much more specific and restricted class of receptors, the Toll-like receptors (TLRs), that can recognize foreign nucleic acids including dsRNAs (67, 68). While the consequence of this remains to be determined, these findings do raise the possibility that RNAi reagents may trigger adverse immune responses in vivo.

USE OF RNAi TO STUDY NORMAL TISSUE PHYSIOLOGY AND DISEASE IN ANIMAL MODELS

In Vivo Delivery of siRNAs to Induce RNAi

By allowing efficient and cheap silencing of gene expression, RNAi promises to provide a significant boost to research of the genetic basis of normal tissue physiology, as well as disease processes in animal models. For this reason, many groups have worked on developing strategies to deliver siRNAs or shRNAs to cells and tissues of experimental animals (Table 1). Early efforts focused on direct administration of synthetic siRNAs, and three major delivery methods have been shown to be successful. The first of these, intravenous injection of siRNAs in a large volume (1 ml) of saline solution, works by creating a back-flow in the venal system that forces the siRNA solution into several organs (mainly the liver, but also kidneys and lung with lesser efficiency) (69, 70).

Gene silencing has also been achieved in vivo by injecting smaller volumes of siRNAs that are packaged in cationic liposomes. When siRNAs are administered intravenously using this strategy, silencing is primarily seen in highly perfused tissues, such as the lung, liver, and spleen (71). Local delivery of siRNAs has been shown to be successful in the central nervous system (72). Finally, gene silencing has also been achieved by electroporation of siRNA duplexes directly into target tissues and organs, including muscle, retina, and the brain (73–76).

Although successful, it is likely that these strategies to silence genes are limited by the stability of siRNAs molecules in vivo and the efficiency with which they are taken up by target cells and tissues. Much effort has been directed to increasing the half-life of the siRNAs by modifying the chemistry of the RNAs used (77–80).

A number of groups have also used plasmid-based shRNAs, instead of siRNAs, to obtain relatively long-lived gene silencing in vivo (81). A number of approaches have also been shown to improve cell and tissue delivery of siRNAs and shRNAs, including conjugating RNAs to membrane-permeant peptides and by incorporating specific binding reagents such as monoclonal antibodies into liposomes used to encapsulate siRNAs (81, 82).

Use of Viral Vectors to Induce RNAi in Primary Cells, Tissues and Experimental Animals

To obtain efficient and long-lived gene silencing using RNAi in cells and tissues, many groups have developed a variety of viral vectors to deliver siRNAs both in vitro and in vivo (Table 1). Retrovirus-based vectors that permit stable introduction of genetic material into cycling cells (83) have been engineered to express shRNAs and to trigger RNAi in transformed cells, as well as in primary cells (58, 84–91). Because they infect and are expressed in certain adult stem cells, notably hematopoietic stem cells, retrovirus-based vectors have also been used to create "knockdown" tissues in mice (86).

Even more wide-ranging applications of RNAi have been reported using recombinant lentiviral vectors (Table 1), because these permit infection of noncycling and postmitotic cells such as neurons (84, 85, 87, 91). Lentiviral RNAi vectors have even been used to generate transgenic knockdown animals by infecting embryonic stem cells or single-cell embryos (87, 89). These animals display expected loss-of-function phenotypes and transmit the RNAi vector to their offspring, suggesting that this technique represents an efficient, low-cost alternative to knockout technologies to study normal tissue physiology and disease processes in a variety of experimental animal systems (87, 89).

Highly effective siRNA delivery systems have also been created that are based on adenoviruses and adenovirus-associated viruses (AAV) (Table 1). Adenoviruses can infect a wide range of cells and have been shown to silence gene expression in vivo (55, 92–94). However, they do not integrate into the genome and tend to induce strong immune responses, which may limit their use in some circumstances. In contrast, AAV does not cause disease in humans (95) and can integrate into the genome of infected cells. Unlike retroviruses and lentiviruses, AAV tends to integrate at a defined location in the genome, thus minimizing the chance of a mutagenic effect of the integrated virus (96, 97). Effective gene silencing mediated by AAV-based vectors has been demonstrated following systemic or tissue-specific injection of viral particles (55, 98, 99).

Creation of Transgenic Animal Models Using RNAi

In addition to the use of lentiviral vectors (87, 89), more traditional transgenesis strategies have been used to successfully create loss-of-function models to study gene function in rodents using RNAi, thus providing another strategy by which RNAi might provide an alternative to creating gene knockout animals

(41, 100–102). Inheritable RNAi transgenesis has been achieved both through expression of shRNAs and long dsRNAs whose expression is restricted to the nucleus or oocyte (41, 100–102). On the basis of this small number of pioneering studies, it appears that RNAi is effective at silencing gene expression in many, if not all, tissues (100). In some instances, attempts to create RNAi transgenic animals by injection of plasmids encoding shRNAs into single cell embryos have been unsuccessful, whereas injection of DNA into blastocysts has succeeded (103). This may reflect a toxic effect of overexpression of siRNAs during early development, possibly due to competition with miRNA pathways. How significant an impediment this might be for the generation of RNAi transgenic animals remains to be determined.

Whereas RNAi in transgenic animals has been shown to recapitulate some loss-of-function phenotypes established in knockout animals, there is mounting evidence that the RNAi phenotype will often appear more variegated than the knockout phenotype (87, 104). This is probably due to the fact that RNAi does not abrogate gene expression but rather reduces it to varying levels. Although this may in some cases limit the use of RNAi in vivo, it is also likely to provide important new insights into the genetic basis of normal tissue physiology, disease processes, and therapeutic strategies by demonstrating the effects of altering gene expression to varying degrees. In particular, RNAi may prove especially important in the creation of animal models of human diseases in which susceptibility and resistance are encoded by alleles that show relative, rather than absolute, differences in expression levels (86).

GENETIC SCREENS USING RNAi

Most researchers use RNAi as a simple reverse genetics tool to understand the function of one or a few genes. On the other hand, RNAi represents an ideal strategy to decipher the role of hundreds or thousands of genes simultaneously in screens for specific phenotypic changes. These forward genetic approaches may help to gain insight into complex physiological and pathological processes.

RNAi Screens in Lower Organisms

The discovery of RNAi and sequencing of the genome of popular model organisms such as *C. elegans* and *D. melanogaster* provided the impetus for functional genetic screens (Table 2). The first such screens were performed in *C. elegans* and focused on easily detectable phenotypic changes, such as viability and sterility, and identified the biological role of a few hundred genes located on chromosomes I and III (105, 106). These studies were significantly facilitated by the finding that RNAi can be induced simply by feeding this worm with bacteria overexpressing dsRNA molecules of interest (107, 108). More recently, genome-wide RNAi screens targeting ~90% of the predicted transcripts have led to the functional annotation of an additional ~2000 genes (109, 110). Importantly, these and other RNAi-based screens have identified the role of genes that have conserved

TABLE 2 Genetic screens using RNAi

Model organism/ system	Delivery method/ type of RNAi molecule	Number of targeted genes	Phenotypic assay/endpoint	References
C. elegans	Feeding with bacteria overexpressing dsRNA library	~17,000 (~90% of known or predicted transcripts)	Viability, sterility, embryogenesis, fat metabolism, genomic stability, mitochondrial function, life span	(105, 106, 109, 111, 112, 114, 173, 174)
D. melanogaster	Soaking cultured cells in dsRNA-containing medium, dsRNAs are typically synthesized by in vitro transcription	~20,000 (~90% of known or predicted transcripts)	Viability, growth, cell morphology, various signaling pathways, innate immune response	(116–119, 123, 124)
Cultured mammalian cells	Transfected siRNA library (chemically synthesized)	510 (1 siRNA per gene)	TRAIL-induced apoptosis	(125)
	Plasmid shRNA library (transfected)	50 (4 shRNAs per gene)	NF-κB signaling pathway	(126)
	Transfected siRNA library (expressed from PCR products, synthesized in vivo)	~8000 (2 siRNAs per gene)	NF-κB signaling pathway	(129)
	Retroviral shRNA library	~8000 (3 shRNAs per gene)	p53-dependent proliferation arrest	(127)
	Retroviral shRNA library	~5000 (3–9 shRNAs per gene)	26S proteasome function	(128)

orthologs in mice and humans and are involved in processes as diverse as genomic stability, fat metabolism, mitochondrial function, and embryogenesis (111–114). They have also provided evidence that RNAi pathways are under genetic control in this organism (115), a discovery that may have significant impact on the use of RNAi both in basic research and in clinical applications.

RNAi can also be induced with ease in cells derived from fruit flies by adding dsRNAs to the culture medium (3). RNAi-based genetic screens have been performed to systematically explore important signal transduction cascades, such as the Wnt-, Hh-, PI3K- and MAPK-pathways, and more broadly to examine the function of most known kinases and phosphatases (116–118). Other high throughput screens identified genes involved in the regulation of cell shape, cytokinesis, and phagocytosis, as well as in heart development and innate immunity (119–123). Recently, Boutros and colleagues targeted almost all of the predicted *Drosophila* mRNAs to analyze their possible roles in cell growth and viability (124).

RNAi Screens in Mammalian Systems

Soon after the basic requirements to create successful RNAi reagents for mammalian systems were established, multiple groups started to assemble si/shRNA

libraries that targeted increasingly larger numbers of genes. These have been successfully applied to study a variety of cellular processes. Initial studies examined the function of a relatively small set of genes in specific signaling or metabolic pathways (125, 126).

Very recently, much larger RNAi libraries attempting genome-wide coverage have been created (Table 2). Generating such libraries and delivering them into mammalian cells has presented real challenges that have been solved in part by using strategies borrowed from technical breakthroughs in the fields of gene sequencing and chemical genetics. Recent reports from Berns et al. and Paddison et al. document the results of the first such large-scale RNAi-based genetic screens in mammalian cells using retrovirus-based vectors to express shRNAs (127, 128). A notable detail of the strategy used by these workers is the inclusion of short unique DNA sequences or the use of the ~60-nt-long shRNA coding sequences as "molecular bar codes" in RNAi vectors. Both groups showed encouraging results that these sequences could be detected using high-density oligonucleotide arrays and thus could circumvent the need to identify shRNAs introduced into cells by sequencing. Zheng and colleagues have also performed a large-scale RNAi-based screen in tissue culture cells using a PCR-based approach to generate siRNAs in vivo (129).

The large-scale genetic screens have successfully identified a number of novel genetic elements of the interrogated signaling or metabolic pathways. On the other hand, certain known components of these pathways were not identified even if the respective targeting constructs were present in the applied RNAi libraries. This discrepancy may be explained by the fact that sometimes not all pathway components can be assessed by the same phenotypic screen. Moreover, despite careful design and use of multiple targeting sequences, some genes may not be effectively silenced to produce the assayed phenotype. However, functional validation of each si/shRNA molecule represents an even bigger challenge than the construction of the library. The recently reported high-throughput methods are based on overexpression of the target gene in fusion with a reporter construct (typically green fluorescent protein; GFP), which require in-frame cloning of the coding sequences of the targeted genes and data handling of hundreds of gigabytes of visual images (130, 131). Therefore, these methods are laborious and expensive and do not allow for the selection of si/shRNA molecules that target untranslated regions of the gene. Currently, other strategies are under development that may surpass the need of in-frame cloning and would also make possible the effective selection of dsRNAs targeting noncoding sequences (P. Sandy, A. Ventura, & T. Jacks, manuscript in preparation).

RNAi should also provide an efficient approach to systematically investigate the genetic basis of normal and disease physiology in animal models. Although RNAi screens performed in vitro have identified a number of physiologically or pathologically relevant genes, these models can often be biased by specific genetic background and/or environmental conditions. Furthermore, many complex physiological or disease processes have no in vitro correlates, such as organ

development or metastasis. These limitations will likely be overcome by performing RNAi screens in vivo once appropriate strategies have been developed.

RNAi IN HUMAN THERAPY

RNAi as a Therapeutic Strategy

The potency and flexibility exhibited by RNAi in experimental systems has stimulated efforts to use RNAi-based reagents in the clinic as "molecular targeting" therapeutics to shut down disease-associated genes in humans (132–134). In theory, therapeutic RNAi should be able to alter the expression and function of genes in a wide range of disease settings. Indeed, RNAi has been used to silence the expression of exogenous disease-causing genes, such as those of pathogens, as well as endogenous genes that play an essential role in the disease process (132) (Table 3). The high degree of specificity of RNAi has even been able to distinguish between alleles of genes exhibiting spontaneous or inherited polymorphisms and alternative splicing events that underlie the development of cancer and other diseases (58, 135) (Table 3).

These findings suggest that the expression of disease-associated genes could be inhibited by RNAi-based reagents. These reagents have been shown to be effective as potential therapeutics in a variety of tissue culture and animal preclinical model systems, including those for cancerous disorders, microbial infections, autoimmune and inflammatory disease, and neurological disorders.

Different siRNAs have recently been described that effectively silence cancer-related genes. These include mutated Ras, Bcr-Abl, and vascular endothelial growth factor (VEGF); the focal adhesion kinase (FAK); Bcl-2; MDR-1; human papillomavirus (HPV) E6 and E7 proteins; CDK-2; MDM-2; PKC-α and β; and TGF-β1 (53, 58, 136–144). Studies in the mouse have shown that injection of siRNAs alone or in combination with anticancer drugs is able to promote apoptosis and reduce

TABLE 3 Exogenous and endogenous disease-associated genes successfully targeted by RNAi

Type of disease	Target	Reference
Viral diseases	HIV (viral genes-genome)	(98, 151, 152, 175–184)
	HIV (cellular receptors/enzymes)	(184–189)
	HBV	(190–192)
	HCV	(69, 147, 148, 193–195)
	HDV	(196)
	Cytomegalovirus (CMV)	(197)

(Continued)

TABLE 3 (*Continued*)

Type of disease	Target	Reference
	Influenza virus	(145)
	Rhinovirus	(198)
	SARS coronavirus	(149)
	Prions	(156)
	Gamma herpes virus	(199)
Autoimmune/inflammatory disorders	TNF-α	(71)
	Fas/CD95/Apo1	(48)
	Caspase-8	(154)
Neurological diseases	Mutated SOD (amyotrophic lateral sclerosis)	(135, 200)
	BACE1 (Alzheimer's disease)	(155)
	SCCMS (myastenic disorders)	(201)
	Polyglutamine proteins	(55)
Cancer/malignant hyperproliferative disorders	Bax	(202)
	CXCR4	(163)
	Focal adhesion kinase (FAK)	(140)
	EphA2	(203)
	Matrix metalloproteinase	(204)
	AML1/MTG8	(205)
	BCR-Abl	(136, 137)
	BRAF(V599E)	(206)
	Brk	(207)
	Epstein-Barr virus (EBV)	(208)
	EGFR	(209, 210)
	Fatty acid synthase (FASE)	(211)
	HPV E6	(143)
	Livin/ML-IAP/KIAP	(212)
	MDR	(142)
	BCL-2	(138, 213)
	CDK-2	(138)
	MDM-2	(138)
	PKC-α	(138)
	TGF-β	(138)
	H-Ras	(138)
	K-Ras	(58)
	VEGF	(53, 138)
	PLK1	(214)
	Telomerase	(215)
	S100A10	(216)
	STAT3	(217)
	NPM-ALK	(218)

tumor burden (139, 140). This provides proof of principle that RNAi for cancer-related genes is feasible in vivo and may prove to be helpful in cancer therapy.

The possibility of using siRNAs to combat infections, especially by viruses, has also been extensively explored recently. Many genes from important human viral pathogens, including HIV, HBV, HCV, influenza virus, and SARS cornavirus, have been shown to be targets for RNAi (69, 134, 145, 146). Inhibiting these viral genes has been shown to interfere with viral replication in vitro (145, 147–149) and in mouse models of viral infection (69, 146, 150). Because many viruses, notably HIV, exhibit high mutation rates (151, 152), RNAi-based therapeutic strategies have also been explored that target host genes that are required for viral entry into cells (134, 153) or that contribute to the pathogenic sequelae of virus infection (154). Similar approaches have also been shown to be successful at modulating inflammatory gene expression in experimental models of immune-mediated diseases (48, 71).

RNAi-based therapeutics can also selectively target mutant forms of genes that underlie the development of neurodegenerative disorders. Inhibition by RNAi of genes encoding proteins involved in polyglutamine-induced neurological disorders (spinocerebellar ataxia type 1 and Huntington's disease) (55, 99), Alzheimer's disease (155), amyotrophic lateral sclerosis (135), and prion-based diseases (156) has been shown and may represent a promising therapeutic strategy.

Delivery Routes for RNAi-Based Therapeutics

Perhaps the most significant barrier for RNAi-based therapy is the efficient and effective delivery of RNAi reagents in patients. As discussed above, a number of strategies have been developed that allow siRNAs and shRNAs to be delivered effectively in animals. Hydrodynamic delivery of siRNAs that involves the intravascular injection of large fluid volumes in order to locally increase intravascular pressure (48, 146) might be adapted for local administration of siRNAs by arterial or venous catheterism in organs, such as liver, kidney, heart or lungs, but cannot be performed for systemic treatment. In the mouse, effective silencing of genes in tumor tissues has been reported following intravenous, intraperitoneal, and subcutaneous injections of siRNAs, suggesting that effective delivery of RNAi reagents may be achieved by different parenteral routes (139).

A number of carrier systems and chemical modifications have been explored to enhance the efficiency and specificity of RNAi-based therapeutics. The use of cationic lipids has been shown to significantly enhance gene silencing (71, 157–159). More sophisticated strategies, in which receptor-specific monoclonal antibodies or other targeting proteins are incorporated into pegylated immunoliposomes (PILs), have been shown to direct gene silencing in a number of tissues, including the brain (81).

Virus-based RNAi delivery systems have also been shown to achieve effective gene silencing in vivo. Systemic or tissue-directed injection of adenoviruses encoding shRNAs has been shown to be effective at inhibiting gene expression in the liver, as well as the central nervous system (55, 91). Retroviruses for RNAi

could potentially be applied for ex vivo cellular manipulations, including those of dendritic cells for the modulation of immune responses (160). However, the use of these vectors may be associated with a risk of insertional mutagenesis and should be carefully evaluated (161) Other virus-based systems for RNAi, such as those based on AAVs, are also being considered as delivery vehicles for therapeutic RNAi (see above).

SUMMARY AND PROSPECTS

In the past few years, RNAi has come to prominence as a novel and essential biological process, as well as a powerful experimental tool and a potential therapeutic strategy. New discoveries in the field of RNAi biochemistry, coupled with technological breakthroughs, have permitted the creation of effective RNAi reagents that can be used to study normal tissue physiology and disease processes in a range of settings, including experimental animals. By further exploring the biology of RNAi and improving delivery and evaluation technologies for RNAi reagents, these strategies will become more effective and more generally available. Now that the first phase I clinical studies of RNAi are on the horizon, several questions related to the safety and efficacy of using RNAi as a therapeutic strategy must be addressed. Ongoing and future preclinical studies in animal models will hopefully help optimize RNAi therapeutics for applications in humans.

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