Published in final edited form as:

Biochem Biophys Res Commun. 2017 April 29; 486(2): 481–485. doi:10.1016/j.bbrc.2017.03.066.

Puromycin-resistant lentiviral control shRNA vector, pLKO.1 induces unexpected cellular differentiation of P19 embryonic stem cells

Jyotshna Kanungo

Division of Neurotoxicology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA

Abstract

RNA silencing is used as a common method for investigating loss-of-function effects of genes of interest. In mammalian cells, RNA interference (RNAi) or RNA silencing can be achieved by transient siRNA (small or short interfering RNA) transfection or by stable shRNA (short hairpin RNA) systems. Various vectors are used for efficient delivery of shRNA. Lentiviral vectors offer an efficient delivery system for stable and long-term expression of the shRNA in mammalian cells. The widely used lentiviral pLKO.1 plasmid vector is very popular in RNAi studies. A large RNAi database, a TRC (the RNAi Consortium) library, was established based on the pLKO.1-TRC plasmid vector. This plasmid (also called pLKO.1-puro) has a puromycin-resistant gene for selection in mammalian cells along with designs for generating lentiviral particles as well for RNA silencing. While using the pLKO.1-puro TRC control shRNA plasmid for transfection in murine P19 embryonic stem (ES) cells, it was unexpectedly discovered that this plasmid vector induced robust endodermal differentiation. Since P19 ES cells are pluripotent and respond to external stimuli that have the potential to alter the phenotype and thus its stemness, other cell types used in RNA silencing studies do not display the obvious effect and therefore, may affect experiments in subtle ways that would go undetected. This study for the first time provides evidence that raises concern and warrants extreme caution while using the pLKO.1-puro control shRNA vector because of its unexpected non-specific effects on cellular integrity.

Keywords

Lentiviral vector; shRNA; ES cells; Endodermal differentiation; Retinoic acid

5. Disclaimer

This document has been reviewed in accordance with United States Food and Drug Administration (FDA) policy and approved for publication. Approval does not signify that the contents necessarily reflect the position or opinions of the FDA nor does mention of trade names or commercial products constitute endorsement or recommendation for use. The findings and conclusions in this report are those of the author and do not necessarily represent the views of the FDA.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2017.03.066>.

1. Introduction

RNA interference (RNAi) or RNA silencing by transient siRNA and stable shRNA systems for loss-of- function studies of genes of interest in mammalian cells have been popularly used in research as well as therapeutic drug discovery and target validation [1–3]. RNAi can be achieved using chemically synthesized double-stranded small/short interfering RNA (siRNA) or vector-based short hairpin RNA (shRNA). The latter are expressed by virus or plasmid vectors. Use of shRNA is intended for more stable gene-knockdown effect in the cells [3]. ShRNA is more advantageous over siRNA because of its relatively low rate of degradation. For conveniently knocking down the gene function in cells by shRNA, “The RNAi Consortium (TRC)” library was built more than a decade ago [4]. This library now contains more than 135,000 lentiviral clones that can target 27,000 genes of humans and mice [5]. The backbone of this large library is the pLKO.1-TRC plasmid. This plasmid not only maximizes viral titers but also resists plasmid recombination. The pLKO.1-TRC plasmid is a third-generation self-inactivating lentiviral vector, in which a human U6 promoter drives the shRNA expression while a PGK promoter drives the expression of the puromycin resistance gene for transduced/transfected cell selection [6]. Lately, a modified pLKO.1 vector tagged to GFP (green fluorescent protein) has been used for convenient selection of cells harboring this vector [7].

Intracellular delivery of genomic materials (transgene or siRNA and shRNA) into specific cells is used not only for therapeutic purposes, but also to effectively alter an existing abnormality within a cell or generate a cell with a new function [8]. To date, thousands of disorders have been treated by more than hundreds of protocols of gene therapy [8]. Through the years, a variety of viral and non-viral vectors for gene delivery has been used and each delivery system carries some advantages and disadvantages. Lentiviral vectors represent one of the commonly used delivery systems for gene therapy with several advantages over other viral vectors. For example, lentiviral vectors have a relatively large transgene (8–9 kb) carrying capacity [9]. Additionally, lentiviral vectors have been shown to have minimal cytotoxicity and immunogenicity [10–12]. Most importantly, these vectors can be integration-deficient, thus avoiding the risk of insertional mutagenesis [13,14]. A number of preclinical studies indicate that lentiviral vector-mediated RNAi-based therapies can have promising outcomes in animal models of CNS disorders [reviewed in Ref. [15]]. However, side effects of siRNA and shRNA as well as the delivering vector itself with off-target effects can pose roadblocks in proper experimental design and developing therapies based on the specific vectors or the siRNA/shRNA sequences.

The P19 embryonic stem (ES) cells were derived from the inner cell mass of a mouse blastoderm and are multipotent cells capable of differentiating to all three germ layers [16]. While these cells are anchorage-independent, lack contact inhibition, and are tumorigenic [17], they respond to a number of morphogens by differentiating into primitive endoderm, mesoderm, neuron-like cells [18] and beating cardiomyocytes [19,20]. The P19 ES cells, therefore, have been used as an ideal model system for studying early embryonic development and differentiation. Exposure of P19 ES cells to low concentrations (10 nM) of all-trans retinoic acid (RA) induces differentiation into primitive endoderm-like cells whereas high concentrations of RA (1 μ M) cause differentiation into neurons and glias

[19,21,22]. A number of gene products and signaling molecules have been shown to be involved in RA-induced P19 ES cell differentiation (reviewed in Kanungo, 2016). RA-induced endodermal differentiation of P19 ES cells requires Gα13 and Gα12 [23–25]. Intracellular signaling pathways involved in this process include the JNK-signaling cascade linking other members of the pathway such as MEKK-1, MEKK-4, and MKK-4 [23–26]. Another study demonstrated that JLP (JNK-interacting leucine zipper protein), a scaffold protein is critical in the RA-induced endodermal differentiation in P19 ES cells [27]. RA-independent endodermal differentiation was shown to be mediated by menin-overexpressing P19 ES cells [28]. In all the above studies, overexpression or knockdown of the signaling molecules in the P19 ES cells were carried out using cDNA plasmid vectors that were either neomycin- or hygromycin-resistant. Those studies showed that the control empty-vector expressing P19 ES cells did not have any effect since those cells sustained their stem cell phenotype. Since P19 ES cells are pluripotent and quickly respond to various proteins and morphogens like RA and DMSO by losing their stemness [reviewed in Ref. [29]], these cells can be used to test the safety of vectors before considering those as carriers of transgenes and shRNAs.

The widely used lentiviral pLKO.1 plasmid vector has been popular in RNAi studies because of the ease of handling, stability and effectiveness. This plasmid (also called pLKO.1-puro) has a puromycin-resistant gene for selection in mammalian cells for RNA silencing along with designs for generating lentiviral particles as well for RNA silencing. In the current study, the puromycin resistant lentiviral control shRNA vector (pLKO.1-puro) [4] was tested in P19 ES cells for its off-target effects, if any. The end point of this safety/toxicity testing was assigned to the retention or loss of stemness of the P19 ES cells.

2. Materials and methods

2.1. Cell culture

The P19 ES cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a humidified atmosphere of 6% CO₂ as reported in earlier studies [28].

2.2. Antibodies and reagents

TROMA-1, a rat monoclonal antibody against cytokeratin Endo A, a marker of primitive endoderm, and a mouse monoclonal antibody (mAb MC-480) that recognizes the stem cell-specific cell surface antigen (SSEA-1), were purchased from the Developmental Studies Hybridoma Bank (University of Iowa, IA, USA). All trans-retinoic acid (RA) was purchased from Sigma (USA) and was dissolved in ethanol (100 mM stock). Puromycin was purchased from Sigma (USA). The plasmid vector pCDNA3, Neomycin (G418) were purchased from Invitrogen (USA). Plasmid harboring non-targeting short hairpin RNA (shRNA), pLKO.1 control vector was purchased from Open Biosystems, USA.

2.3. Plasmids and stable transfection

As previously reported [28], P19 ES cells were transfected with pCDNA3-Neo (Invitrogen, USA) plasmid vector followed by neomycin selection, three stable clones each were isolated. Neomycin (G418) selection was with 400 µg/ml concentration and stable clones were maintained in 100 µg/ml concentration. The pLKO.1-puro (control TRC shRNA vector) plasmid was used to transfect the P19 ES cells. Cells transfected with the pLKO.1-puro plasmid were selected with 4 µg/ml puromycin and stable clones were maintained in 1 µg/ml puromycin. Three stable clones were isolated. Transfection was performed using Lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions.

2.4. Induction of P19 ES cell differentiation

P19 ES cells cultured as monolayers on tissue culture plates in the Dulbecco's modified Eagle's medium (Invitrogen, USA) with 10% fetal bovine serum (Invitrogen, USA) were induced to primitive endoderm by the addition of 10 nM all-trans retinoic acid (RA) 4 days and then cultured for two additional days without RA.

2.5. Indirect immunofluorescence assay

The staining of the embryonic antigen SSEA-1 (with a monoclonal antibody) and the endoderm-specific marker antigen cytokeratin Endo A or TROMA-1 (with a monoclonal antibody), was performed. P19 ES cells were cultured, stained, and subjected to analysis by epifluorescence microscopy as described previously [25,30].

3. Results

3.1. Retinoic acid (RA) induces endodermal differentiation of P19 ES cells

P19 ES cells were treated with 10 nM RA and the control sets of cells were treated with equivalent amounts of ethanol (vehicle). RA at 10 nM concentrations induced robust endodermal differentiation of P19 ES cells cultured in monolayers (Fig. 1). The results showed that in monolayer cultures, vehicle-treated P19 ES cells retained the SSEA-1 antigen (Fig. 1, upper panel) but when treated with 10 nM RA, the cells differentiated into the primitive endoderm type (TROMA-1 positive) and lost the stem cell-specific SSEA-1 antigen expression (Fig. 1, lower panel). The cell nuclear staining with DAPI is shown for both vehicle-treated and 10 nM RA-treated cells (Fig. 1, upper and lower panels). These results reproduced previously established findings that P19 ES cells cultured in monolayers lose their stemness and acquire a primitive endodermal phenotype in response to low dose RA.

3.2. P19 ES cells harboring the pLKO.1-puro shRNA control plasmid differentiate into primitive endoderm

Negative pLKO.1 shRNA control plasmid is an essential part of a properly-designed gene silencing experiment. The TRC vector, pLKO.1-puro, a non-target shRNA control plasmid DNA is a lentivirus plasmid vector. This vector contains a shRNA insert that does not target any known genes from any species, thus making it useful as a negative control in experiments that use the TRC shRNA library clones. The pLKO.1-puro plasmid has the

ampicillin and puromycin antibiotic resistance genes for selection in bacterial or mammalian cells, respectively.

Surprisingly, stably-transfected P19 ES cells harboring the pLKO.1-puro plasmid were TROMA-1-positive indicating that the cells differentiated into an endodermal phenotype (Fig. 2, lower panel). These cells lost the stem cell-specific marker, SSEA-1 (Fig. 2, lower panel). In contrast, P19 ES cells harboring the pCDNA3 plasmid vector (neomycin-resistant) retained the stem cell-specific marker, SSEA-1 and did not express the primitive endoderm marker, TROMA-1 (Fig. 2, upper panel). The cell nuclear staining with DAPI is shown for cells harboring either pCDNA3-Neo or pLKO.1-puro plasmids (Fig. 2, upper and lower panels). These results demonstrated that pCDNA3 plasmid vector is safe and does not have any effect on the P19 ES cells, whereas the pLKO.1-puro alters the cellular integrity with the loss of stemness and acquisition of a primitive endoderm phenotype.

4. Discussion

P19 ES cells are pluripotent and respond to various differentiating agents by acquiring the characteristics of the three primitive germ layer-like lineages [16,18,31]. When treated with low dose (10 nM) RA, P19 ES cells cultured in monolayers undergo endodermal differentiation [18,19]. Previously, it has been shown that P19 ES cells retain their stemness when transfected with various plasmid vectors alone that are neomycin- and hygromycin-resistant [25,26,28,30,32]. In compliance, this study shows that the pCDNA3 plasmid vector which is neomycin-resistant does not alter the P19 ES cell phenotype.

Lentiviral vectors have also been shown to be safe and do not elicit significant immune response [10–12], although some studies suggest that lentiviral vectors may induce an interferon (IFN) response leading to reduced neuronal cell viability [33,34]. Interestingly, when the lentiviral vectors encoded a shRNA, enhanced cytotoxicity was observed [34]. These studies attributed the toxicity to increased vector concentration and recommended to determine the lowest concentration of vectors to be used to produce desired knockdown for functional effects [35,36]. How exactly lentiviral vectors trigger an immune response followed by neuronal cell cytotoxicity is not understood. Nonetheless, it is important to determine the safety and inertness of the lentiviral vectors before they are used in experiments or therapeutic designs.

Due to specific target recognition by the siRNA and the target mRNA that even distinguishes sequences differing by a single nucleotide, siRNAs are considered to be ideal for knocking down gene function. A number of studies, however, have raised concerns over siRNA specificity and the potential for non-specific, off-target effects, even in some cases lethality [35,37–39]. Various microarray studies also showed that siRNAs can alter the expression of a large number of “off-target” genes [37,40]. The off-target effects occurred even before silencing of the target gene [41]. Increase in siRNA concentration in cells was shown to be coincident with the expression of OAS1 [33] and protein kinase R (PKR) [42]. PKR autophosphorylation increased with increasing siRNA concentration, while PKR-null cell lines lacked in the IFN response to siRNA transfection [43].

Upregulated expression of PKR and OAS1 in cultured CNS neurons was shown after lentiviral vector mediated shRNA expression coinciding with reduced expression of off-target genes as well as reduced cell viability [34]. High-level expression of exogenous shRNAs can saturate the endogenous RNA silencing machinery and outcompete the endogenous miRNAs [35] that can cause global disruption in gene expression contributing to cytotoxicity [35,38]. For all these reasons, it is important to determine the lowest viral vector concentration for efficient gene silencing in order to avoid nonspecific cytotoxicity [35,36]. Use of weaker promoters, such as the H1 and 7SK promoters that produce lower levels of shRNA expression that can mitigate the shRNA-mediated toxic effects without significantly reducing silencing efficiency has also been suggested [35,44]. How much of these effects are attributed to the lentiviral vector alone is not clear. All of these studies used biochemical and/or genomic studies to show the off-target effects. These off-target effects did not alter the cellular phenotype to be observable. This study provides an observable endpoint which easily distinguishes the changes in cellular phenotype and therefore provides a simple but feasible method to determine vector safety.

With the results of this study, however, it is difficult to determine whether the pLKO.1-puro vector sequence itself was responsible for inducing the P19 ES cell differentiation or all puromycin vectors have the ability to induce such a response. In human cell lines, it has been shown that puromycin vectors cause an unexpected misfolded protein response and involve reactive oxygen species (ROS) in inducing the formation of nuclear aggresome like structures that can compromise the safety of vector delivery systems [45]. More studies are needed to confirm whether puromycin vectors cause unexpected off-target effects in cells. It is pertinent to test those in the cell types that are amenable to responding to the external agents (e.g., vectors) in a simple manner by changing their phenotype. In this context, P19 ES cells present an advantage as shown in the current study that provides a simple differentiation assay to determine vector safety. Although there have been steady progress in our understanding of RNAi technology, issues still remain regarding the design, use, and side effects of shRNAs. But what is of utmost importance is to ensure that the vector delivering the shRNAs is not intrinsically toxic having off-target effects. Even for third-generation, integration-deficient lentiviral vectors that are one of the leading gene therapy delivery systems, it is essential that adequate tests be undertaken to assure vector safety and neutrality. This study provides for the first time, a time-efficient and reproducible method to test for the safety of lentiviral vectors, such as the pLKO.1-puro, since shRNA-mediated silencing using these vectors shows great promise not only in laboratory studies but also in therapeutic strategies.

References

1. Benson MD, Kluve-Beckerman B, Zeldenrust SR, Siesky AM, Bodenmiller DM, Showalter AD, Sloop KW. Targeted suppression of an amyloidogenic transthyretin with antisense oligonucleotides. *Muscle Nerve*. 2006; 33:609–618. [PubMed: 16421881]
2. Silva J, Chang K, Hannon GJ, Rivas FV. RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. *Oncogene*. 2004; 23:8401–8409. [PubMed: 15517022]

3. Wiederschain D, Wee S, Chen L, Loo A, Yang G, Huang A, Chen Y, Caponigro G, Yao YM, Lengauer C, Sellers WR, Benson JD. Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle*. 2009; 8:498–504. [PubMed: 19177017]
4. Moffat J, Grueneberg DA, Yang X, Kim SY, Kloepper AM, Hinkle G, Piqani B, Eisenhaure TM, Luo B, Grenier JK, Carpenter AE, Foo SY, Stewart SA, Stockwell BR, Hacohen N, Hahn WC, Lander ES, Sabatini DM, Root DE. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell*. 2006; 124:1283–1298. [PubMed: 16564017]
5. Root DE, Hacohen N, Hahn WC, Lander ES, Sabatini DM. Genome-scale loss-of-function screening with a lentiviral RNAi library. *Nat Methods*. 2006; 3:715–719. [PubMed: 16929317]
6. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet*. 2000; 25:217–222. [PubMed: 10835641]
7. Xu J, Zhu W, Guo Y, Jiang C, Hussain N, Meng L, Lu S. Construction of conveniently screening pLKO.1-TRC vector tagged with TurboGFP. *Appl Biochem Biotechnol*. 2016
8. Stone D. Novel viral vector systems for gene therapy. *Viruses*. 2010; 2:1002–1007. [PubMed: 21994667]
9. Frecha C, Szecsi J, Cosset FL, Verhoeven E. Strategies for targeting lentiviral vectors. *Curr Gene Ther*. 2008; 8:449–460. [PubMed: 19075628]
10. Abordo-Adesida E, Follenzi A, Barcia C, Sciascia S, Castro MG, Naldini L, Lowenstein PR. Stability of lentiviral vector-mediated transgene expression in the brain in the presence of systemic antivevector immune responses. *Hum Gene Ther*. 2005; 16:741–751. [PubMed: 15960605]
11. Naldini L, Blomer U, Gage FH, Trono D, Verma IM. Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci U S A*. 1996; 93:11382–11388. [PubMed: 8876144]
12. Schambach A, Zychlinski D, Ehrnstroem B, Baum C. Biosafety features of lentiviral vectors. *Hum Gene Ther*. 2013; 24:132–142. [PubMed: 23311447]
13. Philippe S, Sarkis C, Barkats M, Mammeri H, Ladroue C, Petit C, Mallet J, Serguera C. Lentiviral vectors with a defective integrase allow efficient and sustained transgene expression in vitro and in vivo. *Proc Natl Acad Sci U S A*. 2006; 103:17684–17689. [PubMed: 17095605]
14. Yanez-Munoz RJ, Balaggan KS, MacNeil A, Howe SJ, Schmidt M, Smith AJ, Buch P, MacLaren RE, Anderson PN, Barker SE, Duran Y, Bartholomae C, von Kalle C, Heckenlively JR, Kinnon C, Ali RR, Thrasher AJ. Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med*. 2006; 12:348–353. [PubMed: 16491086]
15. Hutson TH, Foster E, Moon LD, Yanez-Munoz RJ. Lentiviral vector-mediated RNA silencing in the central nervous system. *Hum Gene Ther Methods*. 2014; 25:14–32. [PubMed: 24090197]
16. McBurney MW, Jones-Villeneuve EM, Edwards MK, Anderson PJ. Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature*. 1982; 299:165–167. [PubMed: 7110336]
17. Martin GR. Teratocarcinomas and mammalian embryogenesis. *Science*. 1980; 209:768–776. [PubMed: 6250214]
18. Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI. Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol*. 1982; 94:253–262. [PubMed: 7107698]
19. Bain G, Ray WJ, Yao M, Gottlieb DI. From embryonal carcinoma cells to neurons: the P19 pathway. *Bioessays*. 1994; 16:343–348. [PubMed: 8024542]
20. McBurney MW. P19 embryonal carcinoma cells. *Int J Dev Biol*. 1993; 37:135–140. [PubMed: 8507558]
21. Edwards MK, McBurney MW. The concentration of retinoic acid determines the differentiated cell types formed by a teratocarcinoma cell line. *Dev Biol*. 1983; 98:187–191. [PubMed: 6345235]
22. Rudnicki MA, Reuhl KR, McBurney MW. Cell lines with developmental potential restricted to mesodermal lineages isolated from differentiating cultures of pluripotent P19 embryonal carcinoma cells. *Development*. 1989; 107:361–372. [PubMed: 2483683]

23. Jho EH, Davis RJ, Malbon CC. c-Jun amino-terminal kinase is regulated by Galpha12/Galpha13 and obligate for differentiation of P19 embryonal carcinoma cells by retinoic acid. *J Biol Chem.* 1997; 272:24468–24474. [PubMed: 9305908]
24. Jho EH, Malbon CC. Galpha12 and Galpha13 mediate differentiation of P19 mouse embryonal carcinoma cells in response to retinoic acid. *J Biol Chem.* 1997; 272:24461–24467. [PubMed: 9305907]
25. Kanungo J, Potapova I, Malbon CC, Wang H. MEKK4 mediates differentiation in response to retinoic acid via activation of c-Jun N-terminal kinase in rat embryonal carcinoma P19 cells. *J Biol Chem.* 2000; 275:24032–24039. [PubMed: 10807916]
26. Wang HY, Kanungo J, Malbon CC. Expression of Galpha 13 (Q226L) induces P19 stem cells to primitive endoderm via MEKK1, 2, or 4. *J Biol Chem.* 2002; 277:3530–3536. [PubMed: 11700306]
27. Kashef K, Xu H, Reddy EP, Dhanasekaran DN. Endodermal differentiation of murine embryonic carcinoma cells by retinoic acid requires JLP, a JNK-scaffolding protein. *J Cell Biochem.* 2006; 98:715–722. [PubMed: 16619266]
28. Kanungo J, Chandrasekharappa SC. Menin induces endodermal differentiation in aggregated P19 stem cells by modulating the retinoic acid receptors. *Mol Cell Biochem.* 2012; 359:95–104. [PubMed: 21833538]
29. Kanungo J. Retinoic acid signaling in P19 stem cell differentiation. *Anticancer Agents Med Chem.* 2016
30. Kanungo J, Pratt SJ, Marie H, Longmore GD, Ajuba a. Cytosolic LIM protein, shuttles into the nucleus and affects embryonal cell proliferation and fate decisions. *Mol Biol Cell.* 2000; 11:3299–3313. [PubMed: 11029037]
31. Bain G, Gottlieb DI. Expression of retinoid X receptors in P19 embryonal carcinoma cells and embryonic stem cells. *Biochem Biophys Res Commun.* 1994; 200:1252–1256. [PubMed: 8185572]
32. Kanungo J, Wang HY, Malbon CC. Ku80 is required but not sufficient for Galpha13-mediated endodermal differentiation in P19 embryonic carcinoma cells. *Biochem Biophys Res Commun.* 2004; 323:293–298. [PubMed: 15351736]
33. Bauer G, Dao MA, Case SS, Meyerrose T, Wirthlin L, Zhou P, Wang X, Herrbrich P, Arevalo J, Csik S, Skelton DC, Walker J, Pepper K, Kohn DB, Nolte JA. In vivo biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Mol Ther.* 2008; 16:1308–1315. [PubMed: 18461052]
34. Hutson TH, Foster E, Dawes JM, Hindges R, Yanez-Munoz RJ, Moon LD. Lentiviral vectors encoding short hairpin RNAs efficiently transduce and knockdown LINGO-1 but induce an interferon response and cytotoxicity in central nervous system neurones. *J Gene Med.* 2012; 14:299–315. [PubMed: 22499506]
35. Grimm D, Wang L, Lee JS, Schurmann N, Gu S, Borner K, Storm TA, Kay MA. Argonaute proteins are key determinants of RNAi efficacy, toxicity, and persistence in the adult mouse liver. *J Clin Invest.* 2010; 120:3106–3119. [PubMed: 20697157]
36. Ulusoy A, Sahin G, Bjorklund T, Aebischer P, Kirik D. Dose optimization for long-term rAAV-mediated RNA interference in the nigrostriatal projection neurons. *Mol Ther.* 2009; 17:1574–1584. [PubMed: 19584816]
37. Bilanges B, Stokoe D. Direct comparison of the specificity of gene silencing using antisense oligonucleotides and RNAi. *Biochem J.* 2005; 388:573–583. [PubMed: 15656792]
38. Khodr CE, Sapru MK, Pedapati J, Han Y, West NC, Kells AP, Bankiewicz KS, Bohn MC. An alpha-synuclein AAV gene silencing vector ameliorates a behavioral deficit in a rat model of Parkinson's disease, but displays toxicity in dopamine neurons. *Brain Res.* 2011; 1395:94–107. [PubMed: 21565333]
39. Scacheri PC, Rozenblatt-Rosen O, Caplen NJ, Wolfsberg TG, Umayam L, Lee JC, Hughes CM, Shanmugam KS, Bhattacharjee A, Meyerson M, Collins FS. Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A.* 2004; 101:1892–1897. [PubMed: 14769924]

40. Persengiev SP, Zhu X, Green MR. Nonspecific, concentration-dependent stimulation and repression of mammalian gene expression by small interfering RNAs (siRNAs). *RNA*. 2004; 10:12–18. [PubMed: 14681580]
41. Jackson AL, Bartz SR, Schelter J, Kobayashi SV, Burchard J, Mao M, Li B, Cavet G, Linsley PS. Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol*. 2003; 21:635–637. [PubMed: 12754523]
42. Read ML, Mir S, Spice R, Seabright RJ, Suggate EL, Ahmed Z, Berry M, Logan A. Profiling RNA interference (RNAi)-mediated toxicity in neural cultures for effective short interfering RNA design. *J Gene Med*. 2009; 11:523–534. [PubMed: 19322910]
43. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol*. 2003; 5:834–839. [PubMed: 12942087]
44. An DS, Qin FX, Auyeung VC, Mao SH, Kung SK, Baltimore D, Chen IS. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther*. 2006; 14:494–504. [PubMed: 16844419]
45. Moran DM, Shen H, Maki CG. Puromycin-based vectors promote a ROS-dependent recruitment of PML to nuclear inclusions enriched with HSP70 and Proteasomes. *BMC Cell Biol*. 2009; 10:32. [PubMed: 19409099]

Fig. 1.

RA induces P19 ES cells differentiation into an endodermal phenotype. P19 ES cells were cultured in monolayers in the presence of vehicle (ethanol) (upper panel) or 10 nM RA (lower panel). Cells were treated for a period of four days and cultured without ethanol or RA for two additional days. Cells were then processed for immunocytochemical staining using the SSEA-1, TROMA-1 antibodies and DAPI. SSEA-1 staining for the stem cell-specific antigen (red), TROMA-1 staining for the primitive endoderm (green) and DAPI staining for nuclei (blue) are shown. The images shown are representative of at least three separate experiments performed on different occasions for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

The pLKO.1-puro lentiviral control shRNA vector induces endodermal differentiation. P19 ES cells from stably-transfected with the pCDN3 plasmid vector (upper panel) and pLKO.1-puro lentiviral control shRNA vector (lower panel) were cultured in monolayers for a period of six days. Cells were then processed for immunocytochemical staining using the SSEA-1, TROMA-1 antibodies and DAPI. SSEA-1 staining for the stem cell-specific antigen (red), TROMA-1 staining for the primitive endoderm (green) and DAPI staining for nuclei (blue) are shown. The images shown are representative of at least three separate experiments performed on different occasions for each treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)