# medicine

## Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer

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MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression by repressing translation of target cellular transcripts. Increasing evidence indicates that miRNAs have distinct expression profiles and play crucial roles in numerous cellular processes, although the extent of miRNA regulation is not well known. By challenging mice with lentiviral vectors encoding target sequences of endogenous miRNAs, we show the efficiency of miRNAs in sharply segregating gene expression among different tissues. Transgene expression from vectors incorporating target sequences for mir-142-3p was effectively suppressed in intravascular and extravascular hematopoietic lineages, whereas expression was maintained in nonhematopoietic cells. This expression profile, which could not be attained until now, enabled stable gene transfer in immunocompetent mice, thus overcoming a major hurdle to successful gene therapy. Our results provide novel in situ evidence of miRNA regulation and demonstrate a new paradigm in vector design with applications for genetic engineering and therapeutic gene transfer.

Recently, a complex network of gene regulation was uncovered, which is mediated by small 21–23-nucleotide noncoding RNAs known as miRNAs<sup>1</sup>. miRNAs act as a guide for the RNA-induced silencing complex (RISC) to repress translation of target cellular transcripts<sup>2</sup>. Molecular analysis has shown that miRNAs have distinct expression profiles in different tissues<sup>3,4</sup>, indicating, together with functional studies<sup>5</sup>, important roles for miRNAs in establishing cell identity<sup>6</sup>. As the targets of most miRNAs have not been identified, however, the extent and level of regulation mediated by individual miRNAs is not well known.

Recent studies have used reporter systems to follow miRNA expression *in situ*<sup>7,8</sup>. Although these studies show tracking of miRNA expression, the degree and robustness of miRNA-mediated suppression was not specifically examined. Here, we set out to address these issues, and develop a miRNA-regulated gene expression system that is responsive to lineage commitment and suitable for *in vivo* gene transfer.

Currently, one of the major barriers to stable gene transfer is the development of transgene-specific immunity<sup>9</sup>. In studies of gene

therapy for inherited diseases, such as the hemophilias, a successful outcome has been precluded by the development of immune responses against the vector and transgene product<sup>10</sup>. Several factors contribute to the induction of an immune response following gene transfer<sup>11</sup>. Chief amongst these factors is the direct expression of the transgene product within professional antigen-presenting cells (APCs) of the immune system<sup>12</sup>.

Previously, we and others have used tissue-specific promoters to target transgene expression to hepatocytes and prevent expression within APCs<sup>13–15</sup>. This strategy reduces the incidence and extent of the transgene-specific immune response. Unfortunately, even when tissue-specific promoters are used, neutralizing antibodies against the transgene product and immune-mediated vector clearance can still be observed<sup>14,16</sup>. This may owe to uptake and cross-presentation of the transgene product by nontransduced APCs and/or off-target transgene expression within APCs. The latter can occur because of nonspecific activity of the reconstituted transgene promoter, or, in the case of integrating vectors, because of promoter-enhancer trapping at insertions near active regions of transcription<sup>17</sup>. Restricting transgene expression to a particular cell type may also decrease the potential efficacy of gene transfer by limiting the pool of cells expressing the transgene.

miRNA regulation, which de-targets rather than targets gene expression and functions at the post-transcriptional level, may provide a unique means for overcoming the limitations of current gene delivery systems. We reasoned that, by preventing transgene expression in hematopoietic lineages while permitting high levels of expression in nonhematopoietic cells, miRNA regulation could enable stable gene transfer in the absence of an immune response.

#### RESULTS

#### miRNA regulation prevents expression in hematopoietic cells

We constructed a miRNA-regulated lentiviral vector by inserting four tandem copies of a 23-bp sequence (mirT) with perfect complementarity to either mir-30a, mir-142-5p or mir-142-3p into the 3'-untranslated region (3'-UTR) of a green fluorescent protein (GFP) expression cassette driven by the ubiquitously expressed phosphoglycerate kinase (PGK) promoter (**Fig. 1a**). This design, using multiple copies of a perfectly complementary target, is intended to

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optimize repression of the transgene in the presence of the miRNA, and is based on an emerging understanding of the rules governing miRNA-mediated regulation<sup>18,19</sup>. We chose mir-142-5p and mir-142-3p because recent reports using northern blot and microarray analysis show that these miRNAs are enriched in hematopoietic cells<sup>3,5</sup>. mir-30a is not expressed in 293T cells and is not enriched in hematopoietic tissues<sup>3,20</sup>, and so it serves as a control for our studies.

We transduced 293T embryonic kidney cells with serial dilutions of the vectors. Vectors containing the miRNA target sequences were as efficiently expressed as the parental LV.PGK.GFP vector (**Fig. 1b**). In contrast, transduction of human U937 monocytes and primary



**Figure 1** Endogenous miRNAs effectively de-target gene expression from human hematopoietic cells. (a) Scheme of the proviral vector form of lentiviral vector modified to carry miRNA target sequences. (b) FACS analysis of 293T (kidney origin), U937 (monocyte origin) and primary dendritic cells (derived from peripheral blood) transduced with dose-matched concentrations of the indicated lentiviral vectors at 3 (data not shown) and 14 d after transduction. A lentiviral vector containing the liver-specific albumin promoter (LV.ALB.GFP) is shown for comparison of off-target activity of this promoter. The histograms are representative of three independent experiments. Vector copies per genome (C/G) were determined by Q-PCR. Untransduced cells are shown in gray. (c) FACS analysis of GFP and ΔLNGFR expression from 293T and U937 cells transduced with closely matched concentrations. Dot plots are representative of two independent experiments.

dendritic cells resulted in more than 100-fold reduction in transgene expression between LV.PGK.GFP.142-3pT and LV.PGK.GFP, even though the number of vector copies per genome was similar. This effect was stable in culture and observed with different vector doses, as well as with different transgenes. miRNA regulation was also observed for LV.PGK.GFP.142-5pT; however, expression was less effectively reduced, reflecting differences in the activity of the two miRNAs. Inclusion of a target sequence for mir-30a did not have an effect on the levels of transgene expression in the cell types tested, and thus indicates that the observed effects on the mir-142T vectors were specific for these mirTs.

To study the mechanism preventing expression, we modified a bidirectional lentiviral vector (Bd.LV)<sup>21</sup> to include mir-142-3pT in the 3'-UTR of the GFP reporter cassette (Fig. 1a). This vector exploits the intrinsic bidirectional activity of the PGK promoter to drive divergent transcription of two transgenes. Transduction of 293T cells showed no differences in the expression of GFP or low-affinity nerve growth factor receptor (ALNGFR) between Bd.LV with or without mirT (Fig. 1c). In transduced monocytes, however, the Bd.LV without mirT expressed both GFP and  $\Delta$ LNGFR, whereas the tagged vector only expressed  $\Delta$ LNGFR. This shows that repression of the tagged transgene occurs at the post-transcriptional level, and not by transcriptional silencing, as silencing of the promoter would have prevented the expression of both transgenes.

Quantitative RT-PCR analysis of mRNA from vector copy number-matched monocytes indicated a 35-fold reduction in transgene RNA between the LV.PGK.GFP and LV.PGK.GFP.142-3pT vectors, whereas in 293T cells, comparable levels of RNA were expressed from the two vectors (Fig. 2a). Correspondingly, in cells transduced by the Bd.LVs, the ratio between the levels of transcripts with or without mir-142-3pT approached 1 in 293T cells, but was 0.04 in monocytes (Fig. 2b). Quantitative analysis was confirmed by northern blot (Fig. 2c) and indicated that the reduction in the expression of GFP occurs by transcript degradation and not through repression of mRNA translation.

#### Activity of mir-142-3p is robust in hematopoietic cells

As little is known about the robustness of miRNA activity, we set out to determine whether there was a threshold of regulation that could be overcome by increasing the vector copies carrying mirTs in target cells. After multiple rounds of transduction of U937 cells, there was only an incremental rise in transgene expression, which was linearly related to the vector copies per genome (**Fig. 2d**). These results show that suppression



**Figure 2** mir-142-3p mediates target mRNA degradation in hematopoietic cells in a robust and efficient manner. (a) Quantitative RT-PCR analysis of GFP expression from 293T and U937 cells transduced by LV.PGK.GFP or LV.PGK.GFP.142-3pT. cDNA is from cells shown in **Figure 1b**. (b) Quantitative RT-PCR analysis of GFP and ΔLNGFR expression from U937 cells transduced with the indicated Bd.LV. The cDNA was taken from the cells shown in **Figure 1c**. All values are reported relative to the level of ΔLNGFR transcripts detected in cells transduced with 10<sup>5</sup> TU/ml Bd.LV. (c) Northern blot analysis of cells transduced by lentiviral vector and Bd.LV with (**Fig. 1b**) or without (**Fig. 1c**) mir-142-3pT. Total RNA (20 µg) was loaded for each sample and probed for GFP. The expected size of the GFP transcript is indicated by arrows for the lentiviral vector (top) and Bd.LV (bottom). (d) U937 cells were repeatedly infected with LV.PGK.GFP.142-3pT to obtain increasing vector content. GFP was measured by FACS analysis. Average vector copies per genome (C/G) for the cell population is indicated. A regression analysis showing the relationship between increasing vector dose and transgene expression for LV.PGK.GFP.142-3pT is included (right). In U937 cells, a single copy of LV.PGK.GFP (bottom) expressed GFP at higher levels than 174 copies per genome of LV.PGK.GFP.142-3pT. (e) The robustness of mir-142-3p-mediated RNA interference was measured by superinfection of U937 cells containing 4 copies per genome of LV.PGK.GFP.mir-142-3pT. GP-CR was used to detect the vector copy number of superinfected cells, and changes in GFP and ΔLNGFR expression were measured by FACS analysis.

was maintained to the same extent for all vector doses tested, and that saturation was not reached even at 174 vector copies per genome.

We next asked whether expressing exogenous sequences carrying mirTs could squelch the endogenous miRNA from its natural targets. As no target mRNAs have been identified for mir-142-3p, we overloaded cells with a second vector carrying the same mirT in a different expression cassette. U937 cells carrying 4 copies per genome of IV.PGK.GFP.142-3pT were superinfected with IV.PGK. $\Delta$ LNGFR.142-3pT, and, even after the introduction of 30 copies of a new vector, there was no increase in the expression of GFP (**Fig. 2e**). In addition, expression of  $\Delta$ LNGFR was suppressed by mir-142-3p (**Fig. 2e**).

Overall, our data suggest that mir-142-3p is not reaction limiting in the RNA interference pathway, and that the introduction of new genetic material, containing mir-142-3pT, should not perturb the natural activity of this miRNA.

### miRNAs can selectively de-target gene expression in vivo

After *in vitro* characterization of our miRNA-regulated lentiviral vector in human cells, we extended our studies to the mouse. Mice express exact homologs of each of the human miRNAs we tested *in vitro*, although their tissue expression patterns have not been established *in situ*<sup>4</sup>. We administered  $2 \times 10^8$  lentiviral vector particles to nude mice. Quantitative PCR (Q-PCR) analysis of the spleen and liver showed similar vector content for all treatment groups (data not shown). Expression profiles, however, differed substantially. Mice treated with LV.PGK.GFP and LV.PGK.GFP.30aT showed a widespread pattern of cell expression within the liver, including Kupffer cells, hepatocytes and endothelial cells (**Fig. 3a,b**). In contrast, mice treated with LV.PGK.GFP.142-3pT had almost undetectable expression of GFP in Kupffer cells, but maintained high levels of GFP in hepatocytes and endothelial cells.

Consistent findings were observed in the spleen of treated mice. In those receiving the LV.PGK.GFP vector, there was a high frequency of GFP<sup>+</sup> splenocytes (>5%), with strong levels of expression, as indicated by FACS analysis (**Fig. 3d**). In comparison, <1% of splenocytes from mice treated with LV.PGK.GFP.142-3pT were GFP<sup>+</sup> and only at low intensity. Immunohistochemical analysis of these mice showed the presence of GFP<sup>+</sup> cells found almost exclusively



bars, 120 μm). LV.PGK.GFP.142-5pT effectively de-targeted GFP expression from the CD45<sup>+</sup> leukocytes (red) but permitted strong GFP expression in the nonhematopoietic stromal cells (CD45<sup>-</sup>) of the marginal zone sinus. (d) FACS analysis of GFP expression from splenocytes of LV.PGK.GFP- and LV.PGK.GFP.142-3pT-treated mice.

in the marginal zone. These cells were not of hematopoietic lineage, as indicated by the negative costaining for the pan-leukocyte marker CD45 (**Fig. 3c**), but were reticular fibroblasts<sup>22</sup>, part of the supporting stroma of the spleen. These results show a new aspect of this approach, in which gene expression can be maintained in a wide variety of cell types, while restricting expression from a particular cellular lineage. Of note, the expression pattern of LV.PGK.GFP. 142-5pT did show some level of suppression in hematopoietic cells, but to a much lesser extent than LV.PGK.GFP.142-3pT. As

both mir-142-5p and mir-142-3p derive from the same pri-miRNA transcript, the observed difference in regulation of the two 142T vectors reflects a different activity of the two miRNAs, probably because of post-transcriptional events, such as the extent of RISC incorporation.

#### mir-142-3p demarcates expression between cellular lineages

To better characterize the expression profile of our vector and, correspondingly, the regulatory activity of mir-142-3p, we generated



**Figure 4** mir-142-3p effectively segregates gene expression between hematopoietic and nonhematopoietic lineages throughout the organs of transgenic mice. (a) FACS analysis of GFP expression in the peripheral blood and bone marrow from representative TgN.PGK.GFP.142-3pT (24 copies per genome) and TgN.PGK.GFP (4 copies per genome) transgenic mice. (b) Immunofluorescence of the indicated organs from these mice. GFP (green) was visualized by direct fluorescence. Hematopoietic lineage cells were marked by CD45 immunostaining (red) in all organs analyzed except for the thymus, where CD3 (red) was used to mark thymocytes. In TgN.PGK.GFP mice, pan-cellular GFP expression was detected in the parenchyma and stroma of all organs. Hematopoietic lineage cells appear yellow because of overlap between CD45 staining and GFP expression. In contrast, GFP expression in PGK.GFP.142-3pT transgenic mice was selectively suppressed in the CD45<sup>+</sup> Kupffer cells (liver), alveolar (lung) and lamina propria (gut) macrophages, which appear red and are indicated by arrows. In the spleen and thymus, GFP expression was also negative in all hematopoietic lineage cells, despite strong expression within the stroma of these organs. Scale bars, 120 µm.



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**Figure 5** miRNA-regulated lentiviral vector enables stable gene transfer in immunocompetent mice. Confocal immunofluorescence analysis of liver and spleen sections from Balb/c mice given  $5 \times 10^8$  TU of the indicated lentiviral vector. Scale bars, 120 µm and 60 µm. (a) GFP (green) was visualized in the liver by direct fluorescence; Kupffer cells, CD8<sup>+</sup> T cells or endothelial cells were detected by staining with F4/80-specific, CD8-specific or CD31-specific (red) antibodies, respectively. The GFP<sup>+</sup> cells of LV.PGK.GFP and LV.ALB.GFP mice were cleared from the liver by 2 weeks, which correlated with the presence of CD8<sup>+</sup> T-cell infiltrates. In contrast, abundant GFP<sup>+</sup> hepatocytes and endothelial cells persisted for more than 120 d (latest time point analyzed) in mice injected with LV.PGK.GFP.142-3pT. Vector content was measured at each time point and the copies per genome (C/G) is indicated. (b) GFP<sup>+</sup> cells in the liver of day 70 LV.PGK.GFP.142-3pT–treated mice had the typical morphology of hepatocytes or were CD31<sup>+</sup> (red) endothelial cells (arrows). Scale bar, 60 µm. (c) H&E staining showing normal histology and absence of mononuclear cell infiltration in LV.PGK.GFP.142-3pT mice at 42 d after infection. Scale bar, 60 µm. (d) Analysis of the spleen at 5 d after infection with the indicated lentiviral vector. GFP expression from the mir142-3pT vector was mainly observed at the marginal zone sinus (MS); some of these GFP<sup>+</sup> cells expressed  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA; red) and were identified as fibroblast-like stromal cells (arrows). Scattered GFP<sup>+</sup> cells, including some CD45<sup>+</sup> hematopoietic cells, were present in the spleen of LV.ALB.GFP mice (arrow). Representative pictures from two mice were analyzed per time point. Original magnification,  $\times 200$ . RP, red pulp; TP3, TO-PRO-3.

transgenic mice using the LV.PGK.GFP.142-3pT vector. We analyzed the peripheral blood of  $F_1$  progeny carrying a range of vector copies per genome (from 4 to 24), and found that expression of GFP was virtually undetectable in all hematopoietic lineages (n = 26; **Fig. 4a** and **Supplementary Fig. 1** online). Moreover, despite bright, pancellular fluorescence throughout the parenchyma of liver, gut and lung, as well as the stromal architecture of the spleen, thymus and bone marrow, we observed no expression of GFP within the hematopoietic lineage cells of these organs (**Fig. 4b** and **Supplementary Fig. 2** online). These results show that endogenous mir-142-3p sharply and robustly restricts transgene expression from hematopoietic lineages.

#### miRNA regulation enables stable gene transfer in mice

Finally, we evaluated the utility of our miRNA-regulated lentiviral vector for systemic gene transfer in immunocompetent adult Balb/c mice. We administered 5  $\times$  10<sup>8</sup> transducing units (TU) /mouse of either LV.PGK.GFP, LV.PGK.GFP.142-3pT or a lentiviral vector expressing GFP under the control of the albumin promoter (LV.ALB.GFP). We examined mice at various times for expression of GFP, a strong neoantigen<sup>23</sup>, in the spleen and liver. In mice treated with LV.PGK.GFP, GFP<sup>+</sup> cells were detected at day 5, but, consistent with our previous findings<sup>14</sup>, by day 14 little or no GFP<sup>+</sup> cells were observed and vector content had declined to almost undetectable levels (Fig. 5a). Clearance of GFP<sup>+</sup> cells also occurred with LV.ALB.GFP, despite expression being predominately confined to hepatocytes. Notably, however, off-target expression from this vector was detected in the spleen, including within a small fraction of hematopoietic cells, and may have had a role in the initiation of immune-mediated vector clearance (Fig. 5d).

In contrast to our findings with LV.PGK.GFP and LV.ALB.GFP, GFP<sup>+</sup> hepatocytes and endothelial cells were present at high frequency in the liver of all LV.PGK.GFP.142-3pT-treated mice at all time points analyzed (>120 d, Fig. 5a,b). Morphometric analysis indicated that between 10% and 20% of hepatocytes were GFP<sup>+</sup> (n = 10), and, notably, the frequency of positive cells remained stable. Vector copies per genome were initially similar for all treatment groups, but by day 14 they rapidly diminished in LV.PGK.GFP and LV.ALB.GFP mice, and were maintained to well-detectable levels in LV.PGK.GFP.142-3pTtreated mice. We observed a slow decline in copies per genome at the longest follow up, but because this decline did not coincide with a decrease in GFP+ hepatocytes, it was probably the result of the replacement of transduced Kupffer cells during normal hematopoietic cell turnover. Despite such extensive expression of GFP in the liver, we did not detect any GFP+ Kupffer cells. Moreover, although we did observe GFP<sup>+</sup> reticular fibroblasts in the marginal zone of the spleen, transgene expression was not detected in hematopoietic lineage cells. Consistent with the sustained GFP expression, we did not observe significant CD8<sup>+</sup> T-cell infiltration or signs of pathology in the liver (Fig. 5c). Overall, these results indicate that using the miRNAregulated lentiviral vector, high-level, stable expression of a neoantigen can be successfully established in immunocompetent mice.

#### DISCUSSION

Here we describe a new gene transfer system that exploits the endogenous miRNA machinery for transgene regulation. By using lentiviral vector-mediated delivery, *in vivo* gene transfer was possible, and, as such, we provide some of the first *in situ* data of miRNA activity in an adult mammal. Similar to studies in lower metazoans<sup>24,25</sup>, we observed miRNA regulation to be highly efficient.

In transgenic mice, as well as in mice intravenously administered lentiviral vectors, we observed consistent mir-142-3p activity in all hematopoietic cells. Adding the mir-142-3pT sequence to a transgene produced up to a 100-fold reduction in transgene expression in hematopoietic lineages, with no effect on expression in nonhematopoietic cells.

We also obtained new evidence of the robustness of miRNA regulation, in both *in vitro* and *in vivo* settings, as we did not detect saturation or squelching of mir-142-3p, even in the presence of numerous copies of an exogenously introduced mirT. The considerable efficiency of miRNA regulation observed with our system is consistent with a catalytic process in which the catalyst, mir-142-3p–RISC, is not rate limiting and the substrate, GFP.142.3pT mRNA, is processed at a constant rate at steady state. In addition, it is also possible that a feedback loop exists that leads to increased expression of mir-142-3p in response to increasing target sequences<sup>26</sup>. Further investigation will be required to determine how widely applicable this level of robustness is to other miRNAs.

In our system, endogenous miRNA regulation provided a better means for preventing vector expression in hematopoietic lineage cells than the use of the hepatocyte-specific albumin promoter. This most probably occurred because post-transcriptional regulation can overcome off-target expression resulting from positional effects of insertion and/or imperfect reconstitution of a tissue-specific promoter. This phenomenon may be akin to one of the proposed natural functions of miRNA regulation, which is to prevent translation of mRNAs that were transcription<sup>18,27</sup>. As such, incorporating miRNA regulation into a vector can provide an important layer of control over transgene expression, whether used with ubiquitous promoters or in conjunction with tissue-specific transcription elements.

By using miRNA regulation to de-target transgene expression from hematopoietic lineages, we were able to prevent immune-mediated vector clearance and enable stable gene transfer, thereby overcoming one of the most significant barriers to clinical gene therapy<sup>9</sup>. Although GFP was used as a transgene, we would speculate, based on previous studies with tissue-specific promoters<sup>13–15</sup>, that our approach should be applicable to the delivery of therapeutic genes, including those encoding Factor VIII or IX. In addition to circumventing the immune response, however, miRNA de-targeting, unlike muscle- or hepatocytespecific promoters, enables transgene expression in a larger population of cells, including endothelium. This will raise the overall production of therapeutic protein and could improve clinical outcome.

The effectiveness by which the miRNA-regulated vectors achieved stable gene expression raises questions concerning the underlying immunological mechanism functioning here. mir-142-3p regulation was able to more effectively prevent transgene expression from occurring in hematopoietic cells than the hepatocyte-specific albumin promoter, and may, therefore, have better prevented T-cell priming by professional APCs. It is also possible that antigen presentation by nonprofessional APCs, such as sinusoidal endothelial cells of the liver, provides a pathway for achieving antigen-specific tolerance<sup>28</sup>. Continued studies will be needed to address these issues.

Overall, it is clear that miRNAs can provide a powerful way to regulate a transgene, and by utilizing this complex network, we demonstrate a new paradigm in vector design that has applications for the study of miRNA biology, as well as important implications for therapeutic gene transfer. It also provides an effective method for constructing vectors capable of divergently regulating two distinct transgenes, as we showed with the bidirectional vector. Through this approach, which allows for combinatorial mirT arrangements, a variety of gene delivery constructs, whether used *in vitro* or *in vivo*, for gene therapy or for animal transgenesis, can be created to achieve sophisticated patterns of gene expression. As we continue to discover new tissue-specific as well as developmental and tumor-specific miRNAs, it will be possible to construct vectors that are conditionally responsive to growth or differentiation and even tumorigenesis.

#### METHODS

Plasmid construction. We obtained the hsa-mir-142 sequences from the miRNA registry<sup>29</sup>. We constructed the 4x.mir-142-5p.Target (142-5pT) by annealing the following oligonucleotides: 5'-GGTAGTGCTTTCTACTT TATGCGATGTAGTGCTTTCTACTTTATGACCGGT-3', 5'-CATAAAGTAGAA AGCACTACATCGCATAAAGTAGAAAGCACTACCGC-3', 5'-GTAGTGCTTT CTACTTTATGTCACGTAGTGCTTTCTACTTTATGGTAC-3', 5'-CATAAAGT AGAAAGCACTACGTGACATAAAGTAGAAAGCACTACACCGGT-3'. We constructed the 4x.mir-142-3p.Target (142-3pT) by annealing the following oligonucleotides: 5'-CTAGAGTCGACTCCATAAAGTAGGAAACACTACACGATTC CATAAAGTAGGAAACACTACAACCGGT-3', 5'-TGTAGTGTTTCCTACTTT ATGGAATCGTGTAGTGTTTCCTACTTTATGGAGTCGACT-3', 5'-TCCATAA AGTAGGAAACACTACATCACTCCATAAAGTAGGAAACACTACAC-3', 5'-TC GAGTGTAGTGTTTCCTACTTTATGGAGTGATGTAGTGTTTCCTACTTTATG GAACCGGT-3'. The 142T contains four copies of a sequence (underlined) designed to be perfectly complementary to the respective miRNA. We subcloned the annealed oligonucleotides into the XbaI and XhoI site of pBluescriptII.KS. We subsequently digested the resulting vector with SacII and KpnI and isolated and ligated the 142T fragment into the SacII and KpnI site of pCCL.sin.cPPT.PGK.GFP.WPRE to create LV.PGK.GFP.142-5pT or LV.PGK. GFP.142-3pT. For construction of LV.PGK.GFP.30T, we digested pCMV. luciferase.miR30 (a gift from B. Cullen, Duke University) with BamHI and XhoI and cloned the mir-30aT insert into pBluescriptII.KS. We subsequently digested the resulting vector with SacII and KpnI and cloned the mir-30T insert into the SacII and KpnI site of pCCL.sin.cPPT.PGK.GFP.WPRE. We cloned LV.PGK. ALNGFR.mir-142-3pT by inserting the 4x.mir-142-3pT into the SalI site of the previously described pRRL.sin.cPPT.PGK.\DeltaLNGFR.WPRE vector. Vector production was carried out as previously described<sup>14</sup>. For details on production, titering and cell culture, see Supplementary Methods online.

Vector copy number quantification. We quantified vector copies per genome by real-time Q-PCR, starting from 100 ng template DNA extracted from mouse tissues or 200 ng template DNA extracted from cell lines. The sets of primers and probe used for the analysis were as follows. For the lentiviral vector backbone, primers were: 750 nmol forward, 5'-TGAAAGCGAAAGGGAAA CCA-3'; 200 nmol reverse, 5'-CCGTGCGCGCTTCAG-3'; 200 nmol probe, 5'-VIC-CTCTCTCGACGCAGGACT-MGB-3'. For mouse genomic DNA Actb (encoding  $\beta\text{-actin})\text{, primers were: 300 nmol forward, 5'-AGAGGGAAA$ TCGTGCGTGAC-3'; 750 nmol reverse, 5'-CAATAGTGATGACCTGGCCGT-3'; 200 nmol probe, 5'-VIC-CACTGCCGCATCCTCTTCCTCCC-MGB-3'. For human genomic DNA TERT, primers were: 200 nmol forward, 5'-GGCA CACGTGGCTTTTCG-3'; 600 nmol reverse, 5'-GGTGAACCTCGTAAGTT TATGCAA-3'; 200 nmol probe, 5'-6FAM-TCAGGACGTCGAGTGGAC ACGGTG-TAMRA-3'. We used serial dilutions of DNA from a transgenic mouse or human cell line with known number of lentiviral vector integrations (determined by Southern blot) for standard curves. We carried out the reactions in triplicate in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). We calculated the copies per genome using the equation: ((ng lentiviral vector)/(ng endogenous DNA))  $\times$  (n° of lentiviral vector integrations in the standard curve).

Gene expression analysis. We carried out reverse transcription on 2 µg total RNA using the random hexamers protocol of the Superscript III first-strand synthesis system for RT-PCR (Invitrogen). We performed quantitative PCR analysis to quantify the concentration of *GFP* mRNA, and we used *Gapdh* expression for normalization. We used two sets of primers and probe. For GFP, 20X Assay on Demand (Applied Biosystems), primers were: forward, 5'-CAGCTCGCCGACCACTA-3'; reverse, 5'-GGGCCGTCGCCGACT-3'; probe, 5'-6FAM-CCAGCAGAACACCCCC-MGB-3'. For *Gapdh*, primers were: 200 nmol forward, 5'-ACCACAGTCCATGCCATCACT-3'; 900 nmol reverse,

5'-GGCCATCACGCCACAGSTT-3'; 200 nmol probe, 5'-TET-CCACCCAGA AGACTGTGGATGGCC-TAMRA-3'. We carried out the reactions in triplicate in an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems). For results presented in **Figure 1a**, RNA values were normalized to GAPDH expression and reported relative to transcripts detected from 293T cells transduced with 10<sup>5</sup> TU/ml LV.PGK.GFP, which was set as the calibrator. For results presented in **Figure 2b**, all values are reported relative to the level of ΔLNGFR transcripts detected in cells transduced with 10<sup>5</sup> TU/ml Bd.LV.

**Flow cytometry.** We grew the transduced 293T cells for at least 14 d before FACS analysis to reach steady-state expression of GFP and to rule out pseudotransduction. Before FACS analysis, we detached adherent cells with 0.05% trypsin-EDTA, washed them and resuspended them in PBS containing 2% FBS. We washed cells grown in suspension and resuspended them in PBS containing 2% FBS. For immunostaining, we blocked  $10^5$  cells in PBS, 5% human serum, 2% FBS for 15 min at 4 °C. After blocking, we added R-phycoerythrin (RPE)-conjugated antibodies (LNGFR-specific or CD45-specific; BD Pharmingen) and incubated the cells for 30 min at 4 °C, then washed and analyzed them by two-color flow cytometry on a Beckman Coulter Cytomics FC500 (Beckman Coulter).

*In vivo* vector administration. We purchased 6–8-week-old nude and Balb/c mice from Charles River Laboratories and maintained them under specific pathogen-free conditions. We administered vector by tail vein injection. All animal procedures were performed according to protocols approved by the Hospital San Raffaele Institutional Animal Care and Use Committee.

**Transgenesis.** We generated transgenic mice (TgN) using lentiviral vectors as previously described<sup>30</sup>. Briefly, female FVB mice were superovulated with a combination of pregnant mare serum and human chorionic gonadotropin. We collected 20–30 embryos per female and microinjected them into the perivitelline space with 10–100 pl of  $5 \times 10^7$  TU/ml lentiviral vector stock on the same day. We immediately implanted the manipulated embryos into the oviduct of pseudopregnant CD1 mice. We extracted DNA from pups via the tail and used it to quantify vector copy number by real-time PCR in founder and F<sub>1</sub> progeny mice.

**Immunohistochemistry.** We fixed tissues in 4% paraformaldehyde, equilibrated them in 20% sucrose in PBS for 48 h at 4 °C, embedded them in OCT and froze them. We postfixed cryostate sections (5- $\mu$ m thick) with paraformaldehyde, blocked them in 5% goat serum (Vector Laboratories), 1% BSA, 0.1% Triton in PBS, and incubated them with either mouse-specific rat F4/80 (Serotec) or mouse-specific CD45, CD31 or CD8 (BD Pharmingen). Fluorescent signals from single optical sections were acquired by a three-laser confocal microscope (Radiance 2100; Bio-Rad).

Note: Supplementary information is available on the Nature Medicine website.

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#### COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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