Stable knockdown of microRNA *in vivo* by lentiviral vectors

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Studying microRNA function *in vivo* requires genetic strategies to generate loss-of-function phenotypes. We used lentiviral vectors to stably and specifically knock down microRNA by overexpressing microRNA target sequences from polymerase II promoters. These vectors effectively inhibited regulation of reporter constructs and natural microRNA targets. We used bone marrow reconstitution with hematopoietic stem cells stably overexpressing miR-223 target sequence to phenocopy the genetic miR-223 knockout mouse, indicating robust interference of microRNA function *in vivo*.

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Recently, microRNA (miRNA) inhibition had been reported in cells overexpressing miRNA target (miRT) sequences complementary to an miRNA seed region, suggesting that miRT sequences can act as miRNA 'decoys', 'sponges' or 'antagomirs'[1–3]. Contrary to chemically modified antisense oligonucleotides, miRT sequence–expressing constructs can be stably integrated into the genome, thus opening up the possibility of creating stable cell lines and transgenic animals that are functionally deficient for a specific miRNA family. We previously reported that miRT sequences can be incorporated into lentiviral vectors to tightly regulate transgene expression in a cell type– and differentiation state–dependent manner[4–6], and found no evidence for saturation of miRNA activity in cells transduced with such miRNA-regulated lentiviral vectors. In this study, we optimized lentiviral vectors for miRT sequence overexpression (**Supplementary Methods** online) and achieved stable *in vivo* miRNA knockdown in the mouse. With this miRT sequence overexpression approach, which is an important supplement to genetic knockouts, miRNA function can be studied in mouse models and in human primary cells.

To investigate the dose requirement for stably saturating miRNA, we transduced U937 monocytes, strongly expressing miR-223 (ref. 6), with a control lentiviral vector (*PGK-GFP*) or a lentiviral vector containing miRT sequences complementary to miR-223 (*PGK-223T*; **Supplementary Fig. 1a** online). Both vectors drove expression of destabilized GFP from the phosphoglycerate kinase (*PGK1*) promoter. In control *PGK-GFP*–transduced cells, the number of *GFP* transcripts and the GFP mean fluorescence intensity increased directly proportionally to the number of integrated vectors (**Fig. 1a** and **Supplementary Fig. 1b**). In *PGK-223T*–transduced cells, we observed lower GFP fluorescence for all vector copy numbers tested, indicating that miR-223 was regulating its target. We quantified the activity of miR-223 by calculating the ratio between GFP mean fluorescence of *PGK-GFP*– and *PGK-223T*–transduced cells (fold repression) at matched vector copy numbers. Note that the control *PGK-GFP* and *PGK-223T* dose-response curves were parallel, showing 20–40-fold repression for all vector doses. These results agree with those in our previous report using a bidirectional vector approach[6], and support the conclusion that saturation of miR-223 activity in U937 cells cannot be achieved, even with 40 integrated copies of *PGK-223T*.

Next we replaced the *PGK1* promoter with the stronger spleen focus forming virus enhancer/promoter (*SFFV*) in both vectors, and transduced U937 cells with the constructs, which we named *SFFV-GFP* and *SFFV-223T* (**Fig. 1b**). Again, fluorescence increased proportionally with the number of integrated vectors (**Supplementary Fig. 1b**). With three copies of *SPFV-GFP* fluorescence in cells was as strong as with 15 copies of *PGK-GFP*, and transduction with *SFFV-223T* repressed fluorescence ~20-fold compared to transduction with *SFFV-GFP*, similar to results with the *PGK* vector. However, the fold repression rapidly decreased with increasing vector integration number, suggesting a saturation of miR-223 activity when miR-223T concentration exceeded a threshold. To define this threshold, we isolated 41 U937 single cell–derived clones containing 1–45 copies of *SFFV–223T*. We then binned clones according to increasing vector content, and used the mean fluorescence at each copy number bin to calculate fold repression (**Fig. 1c**). The GFP reporter was suppressed more than 20-fold in clones with ≤5 copies and 5–10-fold in clones containing 6–11 copies of *SFFV-223T*. In clones with ≥12 vector copies, miR-223 was completely saturated. This corresponds to ~20,000 miR-223T sequences, which are required to stably saturate miR-223 in a U937 cell that contains ~16,000 miR-223 molecules[6] (**Supplementary Fig. 1b,c**).

We then constructed additional *SFFV*-driven lentiviral vectors targeting miR-142-3p, miR-16 and miR-23a (*SFFV-142T*, *SFFV-16T* and *SFFV-23aT*, respectively). miR-142-3p was expressed to similar levels as miR-223 in U937 cells, whereas miR-16 and miR-23a were expressed at ~3,000 and ~1,500 copies per cell, respectively[6]. Also these miRNAs lost target suppression activity at high copy number of the corresponding *SFFV*-miRT vector (**Fig. 1d**). However, there were clear differences in the

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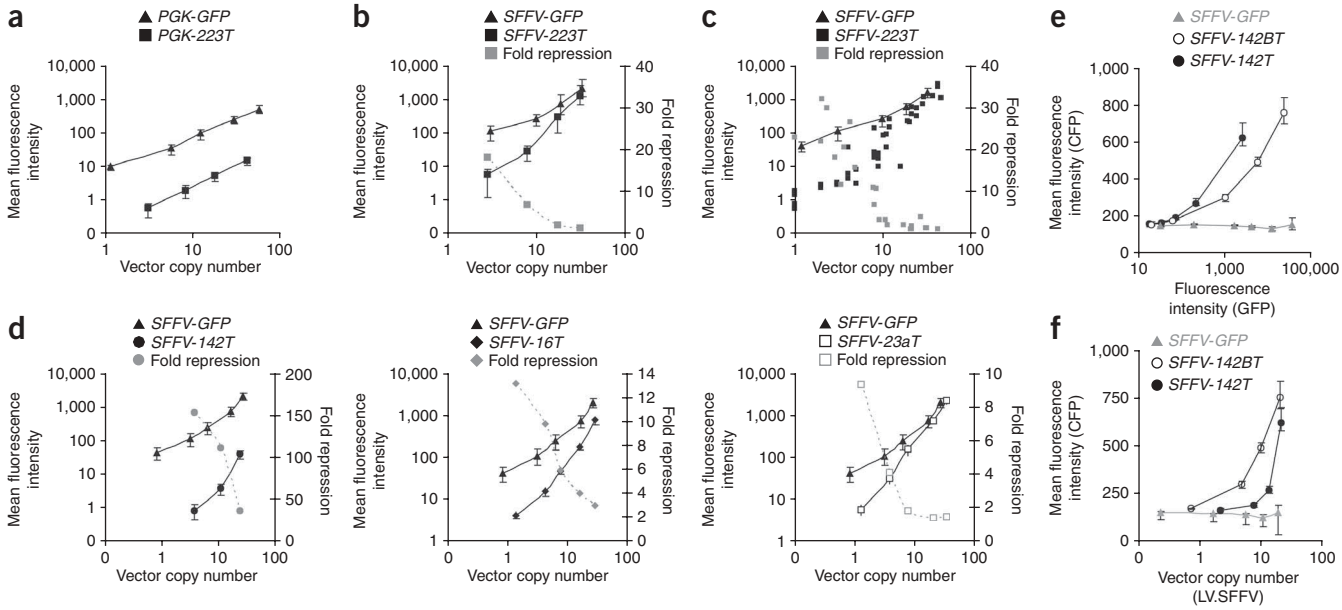

Figure 1 | Saturation of endogenous miRNA activity by stably overexpressing miRT sequences. (**a**) Fluorescence intensity and vector copy number for *PGK-GFP* and *Pgk-223T* (mean ± range, $n = 3$). (**b**) As in **a**, but GFP and 223T were expressed from an *SFFV* promoter (mean ± range, $n = 3$). Fold repression of *SFFV-223T* over *SFFV-GFP* is indicated. Graphs are representative of 4 experiments. (**c**) Mean fluorescence intensities for 41 U937 *SFFV-223T* clones, and fold repression (8 data points from clone bins and 14 data points from *SFFV-223T*–transduced bulk populations) over *SFFV-GFP* mean ± range, $n = 3$; plotted line. (**d**) Dose-response curves for miR-142T, miR-16T and miR-23aT (mean fluorescence intensity ± s.d., $n = 6$) and fold repression. (**e**) GFP and CFP mean fluorescence intensities of U937 cells transduced with equal doses of the *PGK-142BT CFP* reporter and increasing doses of *SFFV-GFP*, *SFFV-142T* or *SFFV-142BT*. (**f**) Knockdown efficiency of these vectors, measured as the loss of regulation (increased fluorescence) of the *PGK-142BT CFP* reporter as a function of *SFFV-142T* or *SFFV-142BT* copy number.

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dose-response relationship. First, each miRNA tested had a different maximal suppressive activity. miR-142-3p reached ~150-fold whereas miR-16 and miR-23a only repressed their targets by 13-fold and ninefold, respectively. Second, the miRNAs were inhibited with varying efficiency by the corresponding miRT. miR-142-3p retained substantial suppressive activity even when challenged with more than 20 integrated copies of *SFFV-142T*, whereas miR-16 had only residual threefold suppressive activity at this vector copy number, and miR-23a appeared to be completely saturated at 8 copies of *SFFV-23aT*. Thus, it appeared that the miRT threshold for saturation varied for each miRNA and positively correlated with its suppressive activity, as measured by the maximal fold repression of the reporter. There was not, however, a strict correlation with miRNA expression. Overall, these data indicate that miRNA activity can be stably saturated in cell lines by titrating the expression of miRT above a concentration threshold using lentiviral vectors carrying strong promoters.

To lower the vector dose required to stably knock down an miRNA, we modified the miRT design. Perfectly complementary miRT can trigger mRNA degradation mediated by a RNA-induced silencing complex (RISC) complex containing Ago-2 ('siRNA pathway')[4], a reaction that is thought to proceed at a high catalytic rate[7]. We[6] and others[1] have suggested that imperfectly complementary, 'bulged' miRT might be more effective in competing with the regulation of natural miRNA targets, which are typically repressed at the translational level ('miRNA pathway'), a reaction that likely proceeds at a slower rate. We thus compared the efficacy of four perfectly complementary miR-142T sequences with efficacies of four bulged miR-142 target sequences (miR-142BT) in saturating miR-142-3p activity in U937 cells when expressed by *SFFV*-driven lentiviral vectors (**Supplementary Fig. 2** online). To directly compare the knockdown efficiency of these two vectors, we developed an assay that would allow us to have the same surrogate marker for miR-142-3p activity. To this end, we transduced U937 cells with a *PGK*-driven cyan fluorescent protein (*CFP*) reporter vector containing four bulged miR-142T (*PGK-142BT*), mimicking natural miR-142-3p target sites. Mean CFP fluorescence was only slightly above autofluorescence (data not shown), indicating suppression of the *CFP* transcript by endogenous miR-142-3p. We then infected this reporter cell line with increasing doses of *SFFV*-driven lentiviral vectors overexpressing miR-142T or miR-142BT linked to GFP (**Fig. 1e**). Increasing doses of *SFFV-142BT* or *SFFV-142T* led to a dose-dependent increase in CFP fluorescence (**Fig. 1f**). Our results demonstrate that *SFFV-142BT* and *SFFV-142T* can saturate regulation of an unrelated transcript (*CFP*) regulated by miR-142-3p. Notably, the dose-effect relationship differed markedly between *SFFV-142BT* and *SFFV-142T*. The miR-142BT sequence caused diminished regulation of the *CFP* reporter with a lower dose dependence than observed for the perfectly complementary miR-142T. However, above vector copy number of 10, both *SFFV-142T* and *SFFV-142BT* caused a similar, near-complete loss of regulation of *CFP*. In summary, these data show that transcripts containing four perfectly complementary miRT sequences can saturate miR-142-3p regulation of perfect and imperfect targets, and thus may effectively squelch the miRNA from its natural targets. However, this only occurs when expression is very high, as can be obtained by using strong promoters and by introducing multiple vector copies. Bulged miRT sequences, in contrast, are more effective than

perfectly complementary miRT at squelching miRNA from bulged targets when assessed at the same doses. We further improved the efficiency in interfering with the regulation of bulged targets by inserting eight bulged miRT sequences instead of four in the context of *SFFV-GFP* (**Supplementary Fig. 2d**).

To determine whether miRT overexpression interferes with the regulation of natural miRNA targets, we measured expression of nuclear factor IA (NFI-A), a known target of miR-223 (ref. 8), in cells transduced with saturating doses of miR-223T. We transduced U937 cells with increasing doses of a lentiviral vector overexpressing four perfectly complementary or eight bulged miR-223 target sequences (*SFFV-223T* and *SFFV-223BT*, respectively). The control vector contained scrambled miRT (*SFFV-scrT*). At the highest dose, a substantial proportion of cells within the *SFFV-223T* group and all cells within the *SFFV-223BT* group expressed GFP to similar levels as the control group, indicating loss of regulation of the miRT-containing transcript (**Fig. 2a**). Western blot analysis of these cells showed that NFI-A expression increased by ~60% and ~100% in the cells transduced with *SFFV-223T* and *SFFV-223BT*, respectively, as compared to untransduced cells or cells transduced with *SFFV-scrT* (**Fig. 2b**). Although the presence of >50 integrated copies of miR-223T driven by the weaker *PGK* promoter did not result in an increase in the amount of NFI-A in U937 cells[6], overexpressing miRT from the *SFFV* promoter results in the loss of regulation of a natural target for that miRNA, thus achieving a functional knockdown of the targeted miRNA. Moreover, these data suggest that bulged miRT sequences are more effective than perfectly complementary ones at interfering with the regulation of natural miRNA targets and that loss of regulation of the *GFP* reporter is a good indicator for miRNA knockdown.

By measuring miRNA concentration in U937 cells saturated by *SFFV-223T*, we noted only a modest decrease in the amount of miR-223 and no decrease in that of unrelated miRNAs (**Supplementary Fig. 3a** online). Functional activity of other miRNAs was also unperturbed in cells saturated by *SFFV-223T*, as proven by suppression of transduced reporter and *HHV1* thymidine kinase suicide genes carrying target sequences for miR-142-3p (**Supplementary Fig. 3b**,**c**). These results demonstrate that overexpressing miRT specifically affects the targeted miRNA rather than saturating the effector pathway. Moreover, miRNA knockdown could be partially relieved by exogenously increasing miRNA concentration (**Supplementary Fig. 3d**), consistent with a model of competitive inhibition of RISC-loaded miRNA by miRT. This model implies that overexpressed miRT do not irreversibly sequester an miRNA, as also indicated by the inability of bulged miRT to interfere with the regulation of a perfect miRT reporter, even at high doses (**Supplementary Fig. 2c**).

We then transduced mouse bone marrow stem and progenitor cells (HSPC) with *SFFV-223T* ($n = 11$) or *SFFV-GFP* lentiviral vectors containing no or unrelated miRT ($n = 18$), and transplanted them into lethally irradiated, congenic recipients. Vector copy number in the repopulated hematopoietic system was high (spleen: 10.2 ± 2.8, $n = 3$) and in a range in which miR-223 activity was clearly compromised *in vitro* (**Fig. 1b**,**c**). Notably, mice transplanted with *SFFV-223T*–transduced cells developed a greater

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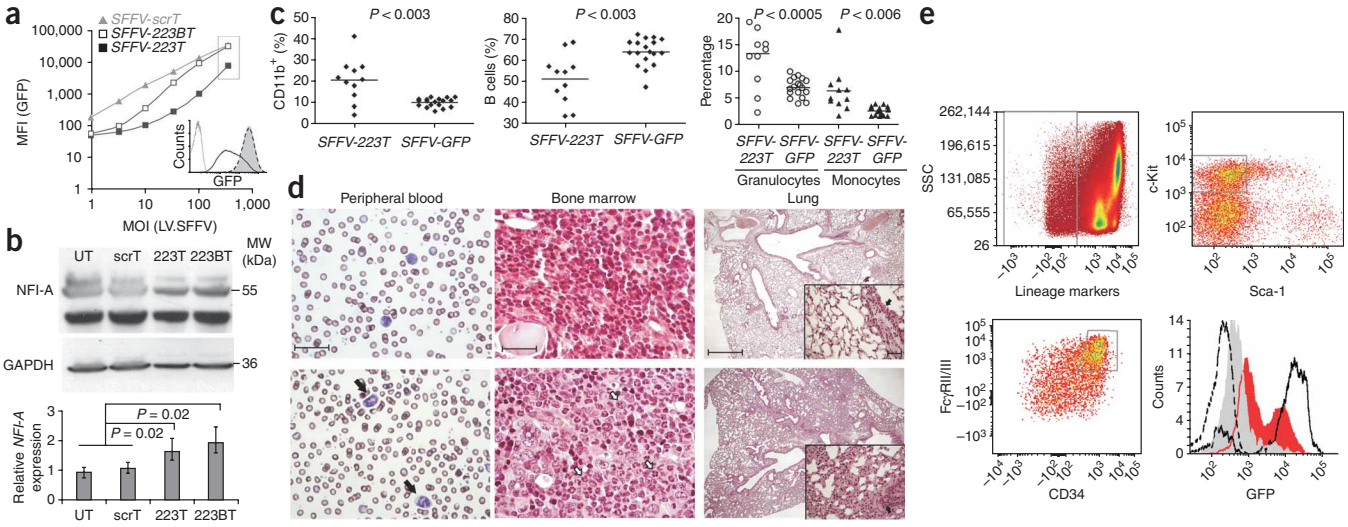

Figure 2 | Inhibition of the regulation of natural miR-223 targets and knockdown of miR-223 activity *in vivo*. (**a**) U937 cells were transduced with increasing multiplicity of infection (MOI) of an miR-223 knockdown vector containing four perfectly complementary or eight bulged miR-223 target sequences and a control vector containing scrambled miRT. Inset, GFP expression at the highest MOI for *SFFV-scrT* (gray filled curve), *SFFV-223BT* (dashed line) and *SFFV-223T* (black line); gray line, untransduced U937 cells. (**b**) Expression of NFI-A in the MOI 300 samples and untransduced cell control (UT) analyzed by western blot. NFI-A protein (55 kDa) relative to GAPDH was quantified by densitometric scanning (mean ± range, $n = 3$; bottom). (**c**) Percentage of myeloid cells ($CD11b^+$), B-cells ($CD19^+$), granulocytes (circles) and monocytes (triangles) in the peripheral blood of mice transplanted with HSPC transduced by *SFFV-223T* ($n = 11$) or by an SFFV control vector (*SFFV-GEP*; $n = 18$) 6–8 months after transplantation. All events were gated on donor cells ($CD45.2^+$). (**d**) Representative PB smear (May-Grünwald-Giemsa staining), bone marrow and lung histopathology (hematoxylin and eosin staining) of a mouse transplanted with *SFFV-GFP*– (top) or *SFFV-223T*–transduced HSPCs (bottom). Note the predominance of granulocytes, often with hypersegmented nuclei, in the peripheral blood (black arrows) and bone marrow (white arrows) of mice expressing *SFFV-223T* (scale bar, 10 μm). Lung overview images (scale bar, 400 μm) and insets (scale bar, 10 μm) show the different extent of septal cellularity between *SFFV-223T* and *SFFV-GFP* mice. Black arrow, bronchus. (**e**) GMP were identified by FACS of bone marrow cells as Lineage $Marker^-Kit^+Sca1^-CD127^-CD34^+Fc\gamma RII/III^+$ cells, and GFP expression is shown for GMP containing *SFFV-223T* (red histogram), *SFFV-GFP* control vector (black line), *PGK-223T* (gray histogram) or no vector (dashed line). FACS plots are representative for 4–5 mice analyzed for each vector.

than twofold increase in the proportion of $CD11b^+$ myeloid cells in the peripheral blood compared to the other mice ($P < 0.003$) and a significant ($P < 0.003$) reduction in the number of B cells (**Fig. 2c**). Numbers of monocytes and granulocytes were significantly increased in the *SFFV-223T* group ($P < 0.006$), but absolute peripheral blood leukocyte counts and hematologic parameters were normal (**Supplementary Fig. 4a** online). Blood smears highlighted an increased prevalence of polymorphonuclear cells in the *SFFV-223T* group, which often appeared hypersegmented (**Fig. 2d**). Bone marrow histopathology revealed absence of neoplastic infiltration and maturing hematopoiesis in the experimental mice, but a clear shift toward myeloid differentiation in the *SFFV-223T* group (**Fig. 2d**). Bone marrow fluorescence-activated cell sorting (FACS) analysis showed a statistically significant increase in the proportion of myeloid cells in *SFFV-223T* mice ($P < 0.02$), while B lymphocytes were decreased, similar to what we found in the spleen (**Supplementary Fig. 4b–d**). Notably, lung histopathology analysis of *SFFV-223T* mice showed marked thickening of the alveolar septa, with increased interstitial cellularity (**Fig. 2d**). This finding diffusely involved the lung parenchyma, and it was specific for the *SFFV-223T* group. A comparison of these results to the recently described miR-223 knockout[9] showed that lentivirus-mediated overexpression of miR-223T in hematopoietic stem cells and their progeny phenocopies important aspects of the genetic miR-223 knockout. Expansion of granulocyte/monocyte precursors (GMP) in the bone marrow underlies the increased frequency of myeloid cells in the knockout mice. Bone marrow FACS analysis in our mice revealed that a high proportion of *SFFV-223T*–transduced GMP expressed the GFP reporter to similar levels as the *SFFV-GFP* control, while GFP was fully and homogenously suppressed in *PGK-223T*–transduced GMP (**Fig. 2e** and **Supplementary Fig. 4e**). These results indicate that *SFFV-223T* saturates miR-223 and likely interferes with the regulation of its natural targets in GMP.

Our findings indicate that lentiviral vectors aimed at knocking down miRNA function should contain a strong promoter, four or more imperfectly complementary binding sites for the targeted miRNA, and should be used at high dose to allow multicopy insertions into primary cells. Such a design may allow simultaneous knockdown of a whole miRNA family[1]. On the contrary, perfectly complementary miRT expressed from moderate cellular promoters are unlikely to interfere with endogenous miRNA function, neither *in vitro* nor *in vivo*, and can thus be used to regulate transgene expression[4–6]. The finding that bulged miRT were inefficient at squelching the miRNA from the regulation of a perfectly complementary miRT supports the notion that stringent suppression of a transgene containing perfectly complementary miRT can be accomplished while the natural targets are being regulated. For this application, one should avoid using miRNA which are members of a larger family, as the same construct may act as a perfect miRT for the targeted miRNA, and as a bulged miRT for related miRNA sharing the same seed, thus running the risk of interfering with the activity of the latter ones.

We envision that these vectors will be invaluable tools to understand the physiological role of miRNA in primary cells and *in vivo*. A potential limitation of our strategy is the need for strong promoters and multiple vector integrations, which increase the risk of insertional mutagenesis in target cells, potentially confounding the identification of miRNA knockdown phenotypes. However, a self-inactivating vector design and the integration pattern characteristic of lentiviruses considerably decrease the genotoxic risk of integration[10]. Furthermore, the possible influence of specific integration sites on the phenotype can be controlled by reproducing the phenotype in different mice, each mouse with a different repertoire of integration sites, and by the lack of appearance of this phenotype in mice carrying a similar load of control vector integrations.

We used the *SFFV* promoter to knock down miRNA in the hematopoietic system. Stable miRNA knockdown in other tissues, including the germline, should be achievable by lentiviral vectors using appropriate, strongly expressed promoters[11]. The use of a polymerase-II promoter to express miRT in the context of a marker gene, which functions as a live reporter for miRNA activity, enables direct assessment of interference with miRNA regulation as well as positive selection of the modified cells. Tissue-specific and drug-inducible expression of the miRT by regulated polymerase-II promoters should allow conditional miRNA knockdown[12,13]. Apart from generating loss-of-function phenotypes, proteomic analysis[14] after miRNA knockdown will allow the identification of key *in vivo* targets that are modulated in the natural setting. Given that miRNA overexpression is associated with pathologic states like cancer[15], stable miRNA knockdown by gene therapy may eventually form the basis of new therapeutics.

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

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AUTHOR CONTRIBUTIONS

B.G. designed and performed research, analyzed data and wrote the paper. G.S., A.G. and M.P. performed research. M.A. and B.D.B. provided crucial reagents. L.N. coordinated the project, analyzed data and wrote the paper.

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