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The Orphan Nuclear Receptor Nurr1 Restricts the Proliferation of Hematopoietic Stem Cells

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Abstract

Successful hematopoiesis requires long-term retention of the quiescent state of hematopoietic stem cells (HSCs). The transcriptional regulation of stem cell quiescence, especially by factors with specific functions in HSCs, is only beginning to be understood. Here we demonstrate that *Nurr1*, a nuclear receptor transcription factor, has such a regulatory role. Enforced expression of *Nurr1* drives early hematopoietic progenitors into quiescence. When stem cells overexpressing *Nurr1* are transplanted into lethally irradiated mice, they home to the bone marrow but do not contribute to regeneration of the blood system. Furthermore, the loss of only one allele of *Nurr1* is sufficient to induce HSCs to enter the cell cycle and proliferate. Molecular analysis revealed an association between *Nurr1* overexpression and upregulation of the cell cycle inhibitor p18, INK4C, suggesting a mechanism by which *Nurr1* could regulate HSC quiescence. Our findings provide critical insight into the transcriptional control mechanisms that determine whether HSCs remain dormant or enter the cell cycle and begin to proliferate.

Keywords

Hematopoiesis; stem cell; quiescence; *p18*; transcriptional control; cell cycle; self-renewal

Life-long hematopoiesis is sustained by the capacity of hematopoietic stem cells (HSCs) to self-renew as well as to replenish mature blood cells. Most HSCs reside in a quiescent, or

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Author Contributions

This study was developed and designed by O.S., who also performed the experiments and wrote the manuscript. G.L. and M.R. contributed to the performance of experiments. O.M.C provided Nurr1$^{-/-}$ mice and discussion. M.A.G. designed experiments and wrote the manuscript.

Competing Interests Statement

The authors declare no competing financial interests.

dormant, state1, a property critical to maintain blood homeostasis. Mutations in a number of genes important for HSC quiescence lead to the loss of hematopoietic repopulating ability2–4. This strong correlation between HSC quiescence and regenerative capacity seems counterintuitive, but suggests that most HSC cell divisions (at least under stress) are symmetric, leading uniformly toward differentiation rather than allowing HSC regeneration. Nevertheless, HSCs are clearly able to self-renew, as demonstrated by single-cell and secondary transplantation experiments5. Identifying the genes whose activities underlie the regulation of self-renewal and differentiation is crucial to realizing the potential of HSCs in regenerative medicine.

The majority of genes known to play roles in HSC quiescence have been those with essential functions in cell cycle regulation in general (e.g. p21, p27, D-type cyclins, RB, p53)4, 6–10, or in transcriptional regulation of hematopoiesis (e.g. Runx1, Egr1 Gfi1)2, 11, 12. To identify candidate genes for a specific role in preservation of quiescence, we distinguished genes that were differentially expressed between dormant and proliferating HSCs13, and uniquely expressed in HSC14; *Nurr1* (aka *Nr4a2*) emerged as a prime candidate. This ligand-independent nuclear receptor-type transcription factor is paralogous to *Nur77* and *Nor1* (aka *Nr4a1* and *Nr4a3*)15–18. While combined loss of *Nor1* and *Nur77* results in an acute myeloid leukemia (AML)-like phenotype in mice19, *Nurr1* has not been implicated in hematopoiesis, although it is known to function in terminal differentiation of dopaminergic neurons20, 21, and regulation of apoptosis22, 23. Thus, the properties of *Nurr1* were sufficiently compelling to warrant its further investigation for a role in HSC quiescence.

Results

Quiescent HSCs proliferate in a synchronized fashion after a single injection of the chemotherapeutic agent 5-flurouracil (5FU)1, 13. Analysis of quiescent versus 5FU-stimulated HSCs revealed ~800 genes whose activation was primarily linked to the quiescent state. One of these, *Nurr1*, was sharply downregulated in proliferating HSCs (Fig. 1a,b), as well as in differentiated hematopoietic cells (Fig. 1c,d). To examine whether *Nurr1* expression in hematopoietic cells is functionally associated with quiescence, we forced its expression in 32D cells, an IL-3-dependent myeloid progenitor line, using a retrovirus carrying *Nurr1* upstream of *IRES-GFP*, allowing us to follow the transduced cells via green fluorescent protein (GFP) (Fig. 1e). An identical vector carrying only *GFP* served as a control. *Nurr1* over-expression led to a striking block in proliferation that inhibited expansion of the transduced 32D cells, while control-transduced cells proliferated readily (Fig. 1f). To ensure that the difference in proliferation was not the result of apoptosis, we performed Annexin V staining. This failed to show any increase in cell death associated with *Nurr1* overexpression (Fig. 1g); the proportion of apoptotic/dead cells in the *Nurr1*-transduced group was significantly lower than in the control group ($p=0.004$), consistent with a pro-survival role of Nurr122. Overexpression of *Nurr1* in 32D cells also led to a decrease in cells positive for the proliferative marker Ki67 (Supplemental Fig. 1a), supporting an inhibitory effect of Nurr1 on proliferation. The Nurr1-associated decrease in proliferation was not associated with increased differentiation of 32D cells (data not shown).

Although supporting the candidacy of Nurr1 as a regulator of cell proliferation, the data presented above do not demonstrate an effect on HSC cycling. Thus, we isolated bone marrow (BM) from 5FU-treated mice (to permit efficient transduction), magnetically enriched for progenitors using the Sca-1 stem cell marker, and transduced them with either of the vectors shown in Fig. 1e (Fig. 2a). After 2 days of culture to allow *GFP* expression, the GFP and Sca-1 double-positive cells were sorted for colony–forming assays, scored on day 14 of growth. The colony-forming ability of *Nurr1*-transduced cells was significantly reduced compared with control cells (4% vs 20%, p=0.002; Fig. 2b), suggesting that Nurr1 impedes proliferation of HSCs. The large decrease in colony number seen with *Nurr1* over-expression could not be attributed to cytotoxicity, as the proportion of viable control and *Nurr1*-transduced cells were identical at the time of plating (data not shown). AnnexinV staining ~20 hours after transduction showed that the proportion of apoptotic cells was about 5% in both the control and *Nurr1*-transduced populations (Fig. 2c). Culture of more highly enriched progenitors for up to 96 hr also resulted in no significant difference in cell viability (Supplemental Fig. 2a), suggesting that Nurr1 overexpression is not toxic to HSCs.

Our *in vitro* data predict that transplanted *Nurr1*-overexpressing cells would not be able to contribute to the replenishment of blood lineages in vivo. To test this, we transplanted *Nurr1*- or control-transduced hematopoietic progenitors into lethally irradiated mice within ~4 hours of transduction, thereby avoiding any effect *Nurr1* overexpression might have on homing (because the protein is not expressed immediately after transduction), and analyzed the peripheral blood of recipients 4 weeks after transplantation for the presence of donor-derived transduced cells, using flow cytometry. Control-transduced cells were found to contribute to about 36% of the peripheral blood, while the contribution of *Nurr1*-transduced cells was only 0.43% (Fig. 2d). Similar levels of nontransduced donor-derived cells were present between infusions of control and *Nurr1*-transduced cells, indicating successful transplantation. Flow cytometric analysis performed 12 months post-transplantation revealed a persistent lack of contribution to peripheral blood from *Nurr1*-transduced donor cells, ruling out delayed engraftment (Supplemental Fig. 2b). With so few Nurr1-over-expressing progeny, we cannot exclude a simultaneous impact of Nurr1 on proliferation and differentiation.

To explain the negligible contribution of *Nurr1*-overexpressing donor cells to peripheral blood reconstitution, we hypothesized that the transduced HSCs home to bone marrow, where they enter the cell cycle at very low levels, if at all. To verify their presence, we examined BM and spleen after transplantation. Nontransduced transplanted cells served as a background autofluorescence control. Four weeks post-transplantation, *Nurr1*-transduced GFP+ cells were present in spleen and BM (Fig 2e). Although these populations were small (<1% of all cells in the tissues), they clearly exceeded the autofluorescent background levels of about 0.1%, indicating their authenticity. The low proportion of Nurr1-overexpressing cells in the BM appears similar to the proportion of transduced cells after initial transplantation. This suggests that control cells were able to proliferate and generate progeny, while the Nurr1-overexpressing cells remained dormant. Since a single HSC can reconstitute a lethally irradiated mouse24, 25, we suggest that a sufficient number of

transduced HSCs were present in the BM to contribute to the reconstitution of peripheral blood; their inability to do so supports our working hypothesis.

To determine whether the proliferative block with *Nurr1* overexpression is reversible, we took advantage of the Cre-lox system. *Nurr1* flanked by *loxP* sites was cloned into the retroviral vector described in Fig. 1e and used to transduce cells expressing *Cre* recombinase under the control of a tamoxifen-inducible promoter (supplemental Fig. 3). Four weeks after transplantation of the transduced cells, tamoxifen was administered to half the group. As expected, mice transplanted with unfloxed *Nurr1*-overexpressing cells or control vector did not respond to tamoxifen treatment: *Nurr1*-transduced cells (measured by GFP) remained at very low levels, while control vector-transduced cells were abundant. However, in mice transplanted with cells harboring the floxed *Nurr1* construct, there was an increase in GFP-expressing cells in peripheral blood 8 weeks after tamoxifen treatment, suggesting removal of the Nurr1-proliferative block with *Nurr1* deletion (Fig. 2f). The appearance of transduced cells only around 8-weeks after tamoxifen treatment supports the view that Nurr1 functions in HSCs rather than committed progenitors to restore depleted blood cell populations, and may suggest the importance of timing of excision as well as the dependency on niche occupancy.

The transplantation data, coupled with the *in vitro* data, indicate that enforced *Nurr1* expression restricts HSC proliferation. To directly determine the cell cycle status of *Nurr1*-overexpressing cells, 48 hr after transduction we sorted and stained GFP-expressing Sca-1 positive hematopoietic progenitors with Pyronin Y and Hoechst 33342 dye, allowing us to distinguish between cells in G_0 vs G_1. Flow cytometric analysis revealed an approximate 50% increase of cells in G_0 with enforced *Nurr1* expression compared to the control (Fig. 3a), supporting an early block of HSC proliferation by Nurr1 activity.

To verify the physiologic relevance of *Nurr1*, we sought to determine whether the loss of *Nurr1* expression would promote HSC proliferation. Homozygous *Nurr1* knockout mice die shortly after birth due to defects in respiratory function26. We therefore selected hematopoietic progenitors (c-Kit+ Sca-1+ Lin− or KSL) from adult WT versus *Nurr1* heterozygous mice (*Nurr1*$^{+/-}$) mice and compared their cell cycle status by Ki67 staining. The results (Fig.3b) indicate that approximately 18% of the *Nurr1*$^{+/-}$ cells were in cycle compared with 12% of the WT cells (p=0.007). Analysis of Sca-1-enriched c-Kit^{+} BM cells with pyronin Y and Hoechst 33342 showed that approximately half as many *Nurr1*$^{+/-}$ compared with WT progenitors were in G_0 (data not shown). As predicted by these results, the colony-forming ability of *Nurr1*$^{+/-}$ HSCs clearly exceeded that of WT cells. Indeed, when BM populations enriched to different purities for HSCs (Sca-1^{+} c-Kit^{+} [SK] vs side population Lin− [SP^{lin-}] vs. side population c-Kit^{+} Lin^{-}Sca-1^{+} [SP^{KLS}]) were plated onto methylcellulose, the colony-forming potential of *Nurr1*$^{+/-}$ progenitors was consistently higher than the control value (Fig. 3c).

If greater numbers of HSCs with reduced levels of Nurr1 enter the cell cycle, they should be more sensitive to killing by antimitotic agents. To test this, we injected *Nurr1*$^{+/-}$ and WT mice weekly with 5FU and then compared death rates between the two groups over 55 days. By 60 days after the first injection, only 2 of 5 WT mice had died, compared with 9 of 10

Nurr1$^{+/-}$ mice (p=0.022, Fig. 3d), supporting our *in vitro* findings. When we examined the HSC population seven days after 5FU injection, we observed a higher level of cell death in the Nurr1$^{+/-}$ HSCs (Supplemental Fig. 4a and b). Thus, the exhaustion of the Nurr1$^{+/-}$ HSCs leading to premature death after repeated 5FU injection is likely due to the higher level of HSC cycling (Fig.3b), but a contribution from higher levels of apoptosis (potentially permitted by Nurr1 loss) cannot be excluded. Thus, both loss- and gain-of-function studies point to a physiologically important role of Nurr1 HSC dormancy.

How might Nurr1 exert its inhibitory effect on HSC proliferation? To begin to address this question, we measured the expression levels of four cell cycle inhibitors (p18 INK4c, p19 INK4d, p21 C.p1/Waf1, and p27 Kip1) in KSL progenitors from the livers of WT, *Nurr1*$^{+/-}$ and *Nurr1*$^{-/-}$ 14.5-day-old embryos, the site of hematopoietic progenitors during this stage of development. The mRNA expression levels of *p18*, *p19*, *p21* and *p27* generally decreased with the loss of *Nurr1* alleles (Fig. 4a). To gain insight into the cell cycle phase affected by Nurr1, we sorted *Nurr1*-transduced HSCs for RT-PCR analysis. Interestingly, *p18*, but not *p19*, *p21* or *p27*, was upregulated in *Nurr1*-overexpressing HSCs (Fig. 4b), although there appeared to be a slight increase in p19 levels in a more heterogeneous progenitor population (Supplemental Fig. 5a). Since p18 and p19 are inhibitors of the cyclin D/CDK4/6 complex needed for cell cycle progression through G_1, while p21 and p27 block later-appearing cyclin/CDK complexes, this indicates that Nurr1 activates cell cycle inhibitors specific for progression through G_1 rather than later cell cycle phases. To confirm upregulation of *p18* with *Nurr1* overexpression at the protein level, we transduced 32D cells with a FLAG-tagged *Nurr1* or control vector. After 4 days of culture (optimal based on earlier experiments; Fig. 1f), protein levels of p18 and p19 were determined by Western blot analysis. The results showed a significant increase (p=0.011) in p18 but not p19 levels with *Nurr1* overexpression (Fig.4c), suggesting that the inhibitory effects of Nurr1 on HSC self-renewal and differentiation may be mediated via p18. This could be direct or indirect activation of p18, as we observed no Nurr1 binding sequence in the p18 promoter.

To determine whether the Nurr1$^{+/-}$ hyper-proliferation phenotype could be relieved by forcibly expressing p18, we expressed p18 from a retrovirus in Nurr1$^{+/-}$ hematopoietic progenitors. Cell cycle analysis demonstrated reduced progenitor proliferation compared to the control (Fig. 4d). Moreover, when p18 expression was forced in Nurr1$^{+/-}$ cells, their proliferative advantage relative to wild-type was lost (Fig. 4e), thereby relieving the Nurr1$^{+/-}$ over-proliferation phenotype. Furthermore, enforced expression of Nurr1 in p18-null cells was not able to cause the proliferation block as it does in WT cells (Supplemental Fig. 5b and c), consistent with a role for p18 downstream of Nurr1.

Here, we show that Nurr1 tightly regulates HSC quiescence. Loss of only a single allele of *Nurr1* in HSCs results in their enhanced cycling and sensitivity to the antimitotic agent 5FU. By contrast, overexpression of *Nurr1* inhibits proliferation of the hematopoietic progenitor cell line 32D, as well as that of BM progenitors plated *in vitro*. Remarkably, *Nurr1* overexpression in transplanted HSCs inhibits their proliferation, but this block is released when *Nurr1* is deleted with the Cre recombinase. Finally, consistent with its role in retaining HSCs in G_0, Nurr1 appears to act via up-regulation of the early G_1-phase cell cycle inhibitor p18.

In general, genes known to play a role in HSC dormancy act broadly in many different tissues. Only a few transcription factors besides *Nurr1* with purported roles in quiescence have expression patterns restricted to hematopoietic progenitors. One of these, the PR-domain-containing transcription factor *Evi1*, is expressed in HSCs at level ~1000-fold higher than in differentiated hematopoietic cells14 and is involved in leukemia generation via chromosomal translocation27. Necdin is another transcription factor that shows much higher expression in HSCs than differentiated hematopoietic cells14, 28, but it appears to have a modest functional role in HSCs4, 28. Importantly, overexpression of *Necdin* fails to impose quiescence in HSC as does *Nurr1* (J. Berg and MAG unpublished).

While *Nurr1* expression appears restricted to hematopoietic progenitors, the paralogs *Nur77* and *Nor1* are expressed in progenitors and myeloid cells. Mice deficient in *Nur77* and *Nor1* KO died 2–4 weeks after birth with an AML phenotype19. Moreover, leukemic blasts from AML patients showed downregulation of *NUR77* and *NOR1*, consistent with a role in the proliferation of hematopoietic progenitors19. As both Nur77 and Nor1 are expressed in mouse HSCs and bind to the same DNA sequence recognized by Nurr129–31, we suggest that all three may cooperate to maintain HSC dormancy.

To identify mechanisms by which Nurr1 may regulate quiescence, we measured expression of four cell cycle inhibitors associated with modulation of HSC proliferation6, 7, 32–34. Only p18 was unequivocally upregulated upon Nurr1 overexpression. Since p18 is thought to preserve HSC function by limiting cell cycle entry34, its regulation by Nurr1 provides a plausible mechanism for the effects of Nurr1 overexpression or deletion seen in this study. Whether Nurr1 targets the p18 gene directly or indirectly remains to be determined.

Because of the importance of Nurr1 in brain development, its role in this tissue has been extensively studied. Recently, it was shown to interact with corepressor for element-1-silencing transcription factor (CoREST), to protect neurons from inflammation-induced apoptosis35. Nurr1 may play a similar role in HSCs during inflammatory stress36. Nurr1 has also been shown to interact with Pitx3, to prevent apoptosis in the brain37, 38. Although not detected in mature hematopoietic cells, Pitx1 is highly expressed in HSCs14, suggesting that common mechanisms may regulate parallel processes in HSCs and developing neurons.

Our studies provide critical insight into the transcriptional machinery that protects HSCs from excessive proliferation that could lead to stem cell exhaustion. Taken in the context of other transcription factors that regulate HSC proliferation such as Gfi1, Mef1/E1f4, GATA2, and members of the FoxO family12, 39, 40, Nurr1 appears to be part of an intrinsic core regulatory circuit that maintains HSC quiescence, much in the way that Nanog, Oct4 or Sox2 control the pluripotency of embryonic stem cells41. Understanding how each of these components functions, and the degree of cross-talk among them, will be crucial to manipulating HSC toward desired therapeutic end-points.

Methods

Retroviral production and transduction of Sca-1$^+$ BM cells

Nurr1 and *Nurr1*-Flag were subcloned into a murine stem cell virus (MSCV), and retroviral particles were generated by cotransfecting a viral vector containing *Nurr1* with *pCL-Eco* into 293Tcells42. The virus particles were collected 48 hr later and frozen. Virus was titered on 3T3 cells to ensure transduction of experimental cells with equivalent moi. Mice were treated with 5FU at150 mg/kg in intraperitoneal injections (with PBS; American Pharmaceutical Partners) and sacrificed 6 days later. Bone marrow cells were collected and enriched for Sca-1+ cells using magnetic enrichment (AutoMACS, Miltenyi). The Sca-1-enriched population was then transduced by spin infection for 2hr at 250 × *g* at room temperature with an appropriate amount of virus 43. Transduction media contained Stempro 34 (GIBCO), penicillin/streptomycin, L-glutamine (2 mM), nutrient supplement, mSCF (10 ng/ml; R&D Systems), mTPO (100 ng/ml, R&D Systems), and polybrene (4 μg/ml; Sigma). After transduction the cells were either incubated for 3hr at 37°C, washed and transplanted into lethally irradiated mice, or cultured further for Annexin V staining or methocult assay.

Proliferation of 32D cells

32D cells were transduced with viral vectors. After 48 hr of culture GFP$^+$ cells were sorted and cultured further. The number of viable cells in each well were counted/scored daily using trypan blue.

Colony forming assay

After 48 hr of culture, GFP$^+$ Sca$^+$ double-positive cells were sorted into 96-well plates containing MethoCult GF M3434 (StemCell Technologies, Inc.) and incubated at 37°C, in 5% CO^2. The numbers of colonies were scored 14 days after plating.

Bone marrow transplantation

Donor bone marrow cells were isolated from 8 to 12-week-old C57Bl/6 (CD45.2) mice. C57Bl/6 (CD45.1) recipient mice were lethally irradiated with a split dose of 10.5 Gy and then transplanted by retro-orbital intravenous injection. Peripheral blood was analyzed at 4, 8, 12 and subsequent weeks to assess engraftment, transduction and lineage distribution.

Annexin staining

Twenty hours after transduction of BM or 32D cells, Sca$^+$ GFP$^+$ double-positive cells were sorted into a carrier population (500,000 B220$^+$ spleen cells), washed with cold PBS, and stained with Annexin V antibody (Becton Dickinson) in Annexin-binding buffer according to the BD manufacturer's protocol. Cells were analyzed with LSRII (Becton Dickinson).

Pyronin Y/Hoechst staining

At 48 hr post transduction, transduced Sca-1$^+$ cells were sorted into a carrier population (500,000 B220+ spleen cells) and stained with 20 μg/ml Hoechst dye and 50 μg/ml Verapamil for 45 min in 37°C, at which time pyronin Y was added to a final concentration

of 1μg/ml and the cells incubated for another 15 min. Samples were washed and analyzed with LSRII.

Survival analysis

Mice were injected weekly with a single dose of 5FU (150 mg/kg) and observed daily for changes in physical health. The times of death were scored and converted to survival plots, with the Gehan-Breslow-Wilcoxon test used to assess statistical significance.

RT-PCR Analysis

After RNA isolation in lysis buffer (GE Healthcare), we performed reverse transcription with random hexamer primers and the Superscript II kit (Invitrogen). The cDNA was then used in PCR amplifications with either Taqman mastermix, an 18 s-rRNA probe (VIC-MGB), and a gene-specific probe (FAM-MGB, Applied Biosystems) or Sybrgreen mastermix and primers targeting the gene of interest as well as Gapdh (for normalization). Reactions were run with an AbiPrism 7900HT (Applied Biosystems) for 40 cycles.

Immunoblotting

Cultured 32D cells were transduced with *GFP*-only or Flag-tagged *Nurr1* and *GFP* expressing MSCV retrovirus. After 96 hr of culture, 6×10^6 GFP-positive cells were solubilized in lysis buffer (PBS, pH 7.4, and 2% IGEPAL, Protease Inhibitor Cocktail, Sigma). The lysates were passed 13 times through a 25-gauge needle and centrifuged at maximum speed for 10 min at 4°C in an Eppendorf microfuge. The clarified lysates were then mixed in even proportions with 2X SDS sample buffer (Bio-Rad) and resolved on 12% Ready Tris-HCl gels (Bio-Rad). The gels were immunoblotted with anti- Flag (Sigma), p18 (Invitrogen), p19 (Invitrogen), and beta-actin (Santa Cruz Biotechnology), monoclonal primary antibodies, and anti-mouse HRP-conjugated (Calbiochem) secondary antibody. Immunoblots were developed with the ECL Plus chemiluminescence reagent (Amersham) and visualized with a Storm 860 phosphorimager or Kodak BIOMAX Light Film (Sigma).

Statistical Considerations

We relied on descriptive statistics (means and standard deviations) to summarize normally distributed data and the two tailed T-test to compare results. Differences with a P value of <0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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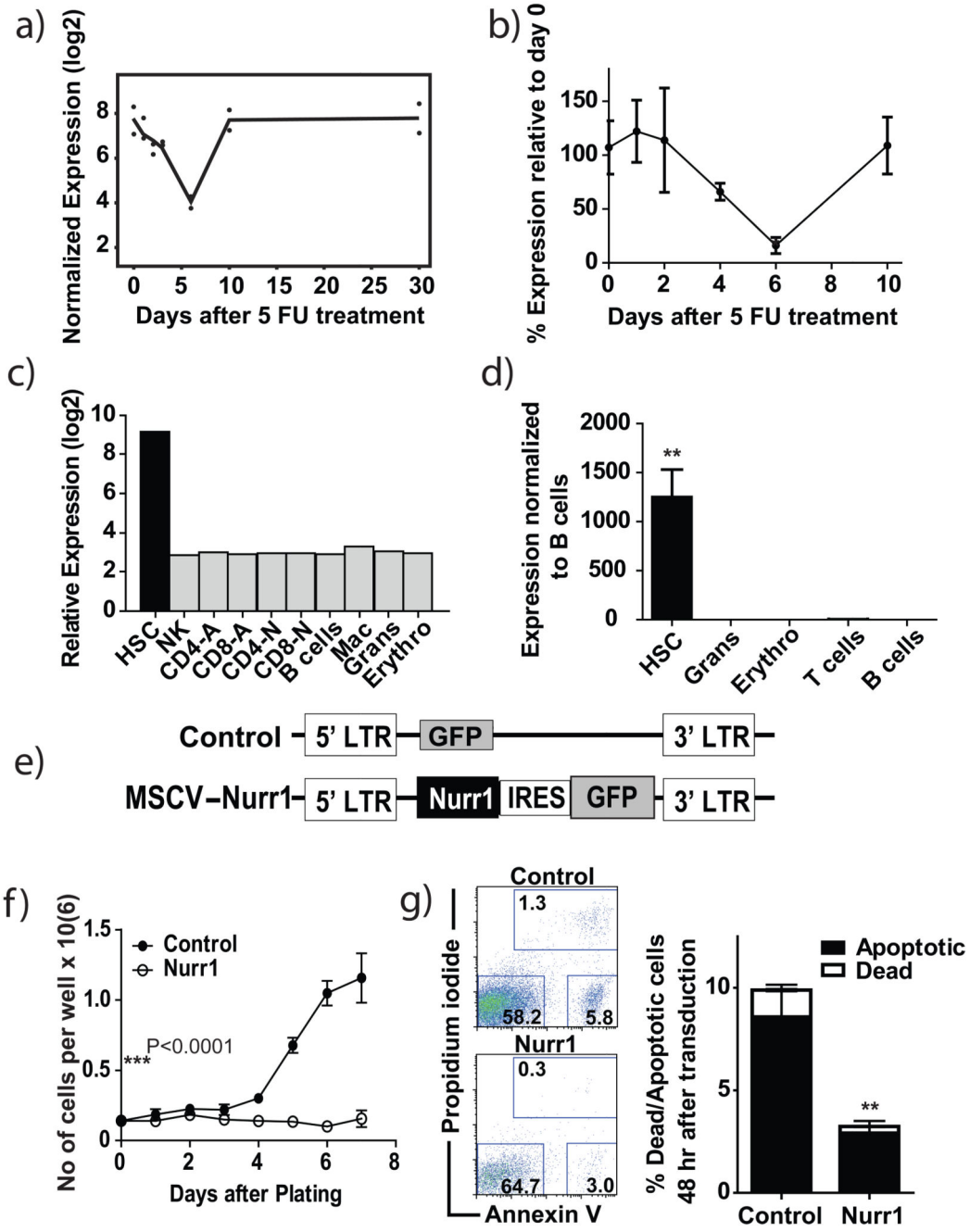

Figure 1. Nurr1 is highly expressed in quiescent HSCs (Hoechst 33342 side population cells that were also c-Kit+, Sca1+, and lineage marker-negative (SP-KSL, or SP^{KLS})) and its over-expression in 32D cells results in a proliferative block

(**a**) Microarray data showing changes in *Nurr1 expression* as the HSC enters cell cycle. (**b**) Real-time PCR verification of microarray data in **a** demonstrating decrease in expression by day 6 (mean of 3 experiments; *p=0.02 between day 0 and day 6). (**c**) Microarray data showing relative expression of Nurr1 in HSCs compared to that of differentiated cells. (**d**) Verification of microarray data by real-time PCR (mean of 3 experiments; p=0.003). (**e**) Vectors used for introducing *Nurr1*. Both vectors are based on MSCV. The control vector

only expresses GFP. MSCV-Nurr1 expresses Nurr1 upstream of IRES-GFP. (**f**) Cells transduced with GFP only control vector are able to thrive, while cells overexpressing Nurr1 do not proliferate (n=3; ***p<0.001) (**g**) but are sustained in the media with no increase in cell death, as determined by Annexin V staining. Left graph is representation of flow data and the quantification is on the right (mean of 3 experiments; **p=0.004). Error bars in panels b,d,f and g denote sem.

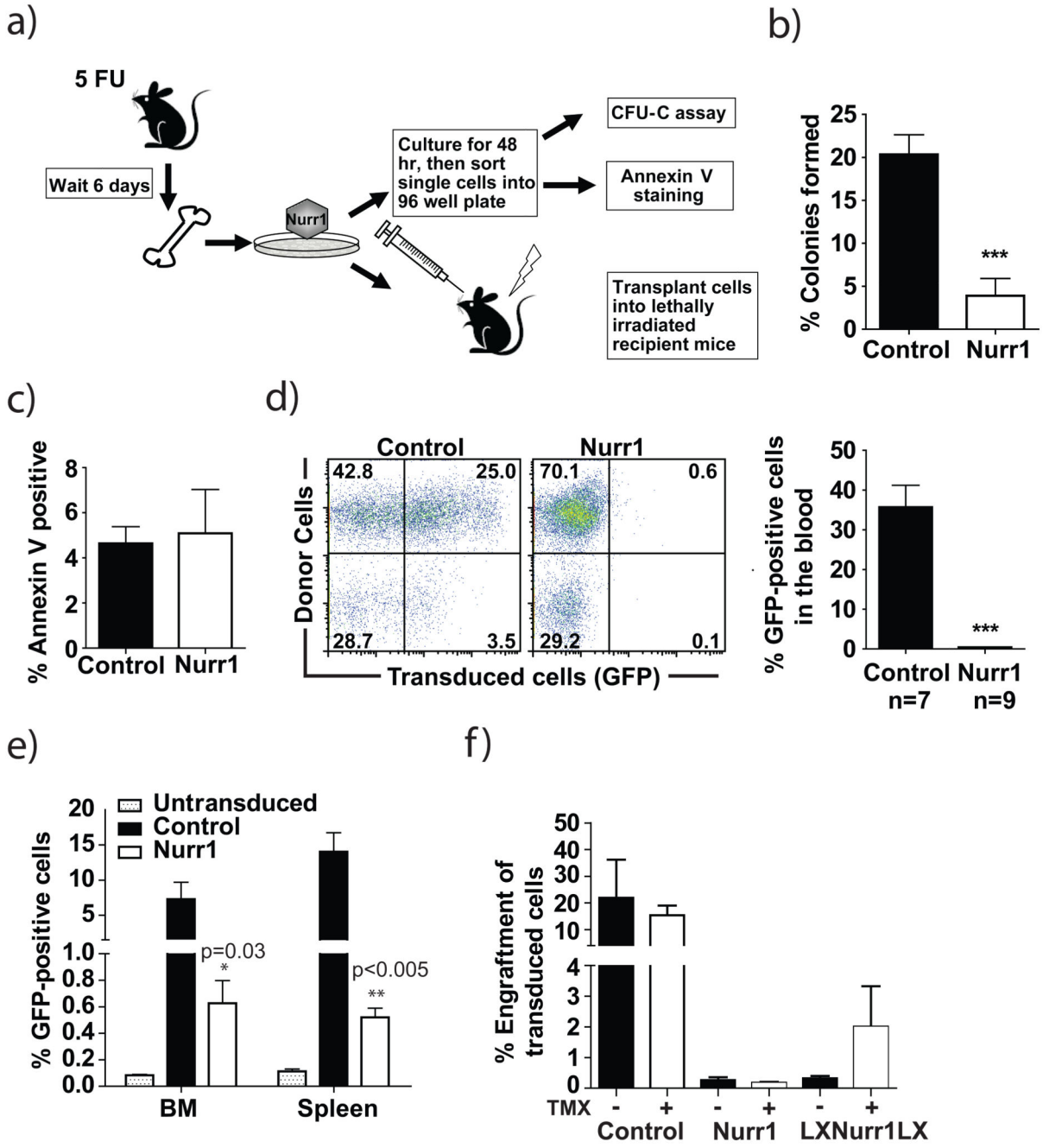

Figure 2. Overexpression of Nurr1 in BM cells reversibly blocks proliferation

(**a**) Schematic of overexpression experiments in BM cells using control and Nurr1 vectors shown in Figure 1. (**b**) Dramatic decrease in number of colonies observed 14 days after plating BM cells onto methylcellulose when overexpressing Nurr1 (n=4; ***p<0.0002). (**c**) Annexin V staining of samples reveals no significant difference in the survival of the cells exposed to control vector or Nurr1 vectors (mean of 5 experiments). (**d**) Left panel is representative peripheral blood analysis 4 weeks after transplantation of control- or Nurr1-transduced BM cells. While ~25% of control cells express GFP, the peripheral blood is

essentially devoid of *Nurr1*-transduced cells. Quantification of contributions are shown in the right panel. This effect persists long-term (Control n=7; Nurr1 n=9; ***p<0.0001). (**e**) Though not contributing to the blood, cells expressing Nurr1 are evident in the bone marrow at low levels (*p=0.03) and spleen (**p<0.005) four weeks after transplantation, as identified by presence of GFP higher than the background-fluorescence levels determined by transplantation of untransduced Sca-1 cells (mean of 3 experiments). (**f**) The proliferation block can be rescued with the removal of enforced *Nurr1* expression, by tamoxifen-induced cre-mediated deletion of the loxP-flanked *Nurr1* gene (vector shown in Supplemental Figure 3). Cells can now proliferate and contribute to the blood, as measured 4-weeks after tamoxifen induction (8 weeks after the initial transplant) (Control n=4; Nurr1 n=4; LXNurr1LX n=6; p= 0.3). Error bars in panels b,c,d,e and f denote sem.

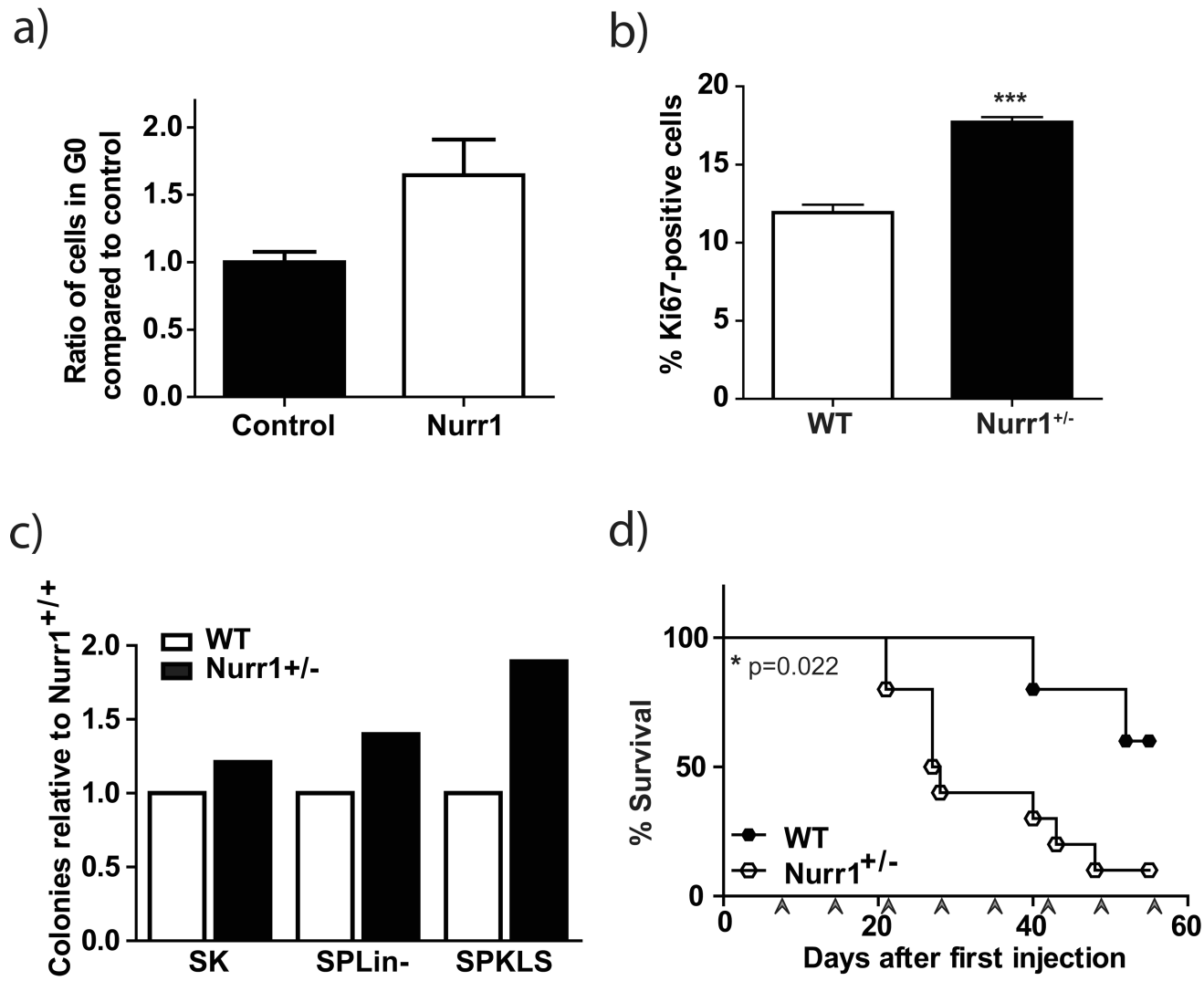

Figure 3. ***Nurr1*** **overexpression leads to reduced cell cycle proliferation**

(**a**) Overexpression of *Nurr1* in BM cells results in decreased number of Sca1+ cells in cycle 48hr post-transduction (control n=5, Nurr1 n=7; p=0.05) whereas (**b**) loss of a single Nurr1 allele leads to an increased proportion of Ki67-positive cells (mean of 3 experiments; ***p=0.0007). (**c**) *Nurr1*$^{+/-}$ HSCs show increased colony-forming ability. SK, Sca-1^{+}c-kit^{+}; SP^{Lin-}, side population Lin^{-}; SP^{KLS}, c-kit^{+}Lin^{-}Sca-1^{+}. (**d**) Post-treatment survival curves of Nurr1$^{+/+}$ (n=5) relative to Nurr1$^{+/-}$ (n=10) mice (*p=0.02 based on Gehan-Breslow-Wilcoxon test). Arrows beneath the graph indicate time of 5FU treatments. Error bars in panels a and b denote sem.

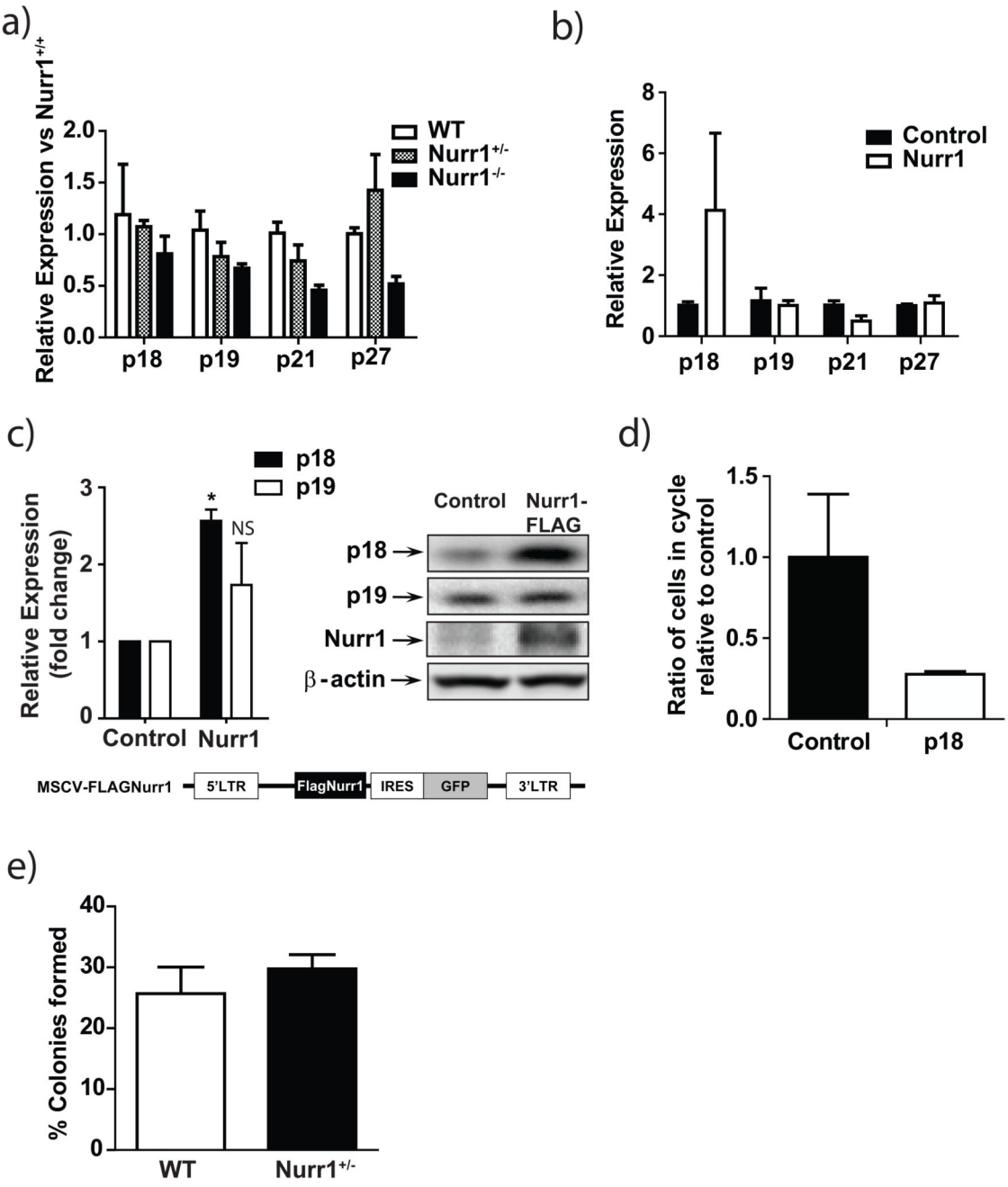

Figure 4. Dose effect of *Nurr1* on expression of cell cycle inhibitors and rescue of Nurr1 phenotype by p18 expression

(**a**) WT, *Nurr1*$^{+/-}$, *Nurr1*$^{-/-}$ E14.5 fetal liver KSL cells (n=3) and (**b**) in Sca-1+ cells transduced HSCs (KSL CD150+ GFP+) with control vector and Nr4a2 vector.(n=4) (**c**) Western showing changes in p18 and p19 with enforced Nr4a2 expression (n=3; *p=0.011) in 32D cells. See full scanned blot in Supplemental Figure 6. (**d**) Nurr1$^{+/-}$ KSL progenitor cells transduced with either a control vector (GFP only) or a vector expressing p18 show different proportions of cells in cycle, with p18 overexpression resulting in a decreased

proportion of cells in cycle (n=3). (**e**) Enforced p18 expression in Nurr1$^{+/-}$ progenitors eliminates the proliferative advantage that Nurr1+/− cells have over wild-type (compare with Figure 3c) (n=3). Error bars in panels a,b,c,d and e denote sem.