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Cyclin C regulates human hematopoietic stem/progenitor cell quiescence

Yasuhiko Miyata[1,3], **Yan Liu**[1], **Vladimir Jankovic**[1], **Goro Sashida**[1], **Jennifer May Lee**[1], **Jae-Hung Shieh**[2], **Tomoki Naoe**[3], **Malcolm Moore**[2], and **Stephen D. Nimer**[1,*]

[1] Molecular Pharmacology and Chemistry Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, USA

[2] Cell Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, USA

[3] Hematology and Oncology Department, Nagoya University, Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, 466-8550 Japan

Abstract

Hematopoietic stem cells (HSCs) can remain quiescent or they can enter the cell cycle, and either self-renew or differentiate. While cyclin C and cdk3 are essential for the transition from the G_0 to G_1 phase of the cell cycle in human fibroblasts, the role of cyclin C in hematopoietic stem/progenitor cells (HSPCs) is not clear. We have identified an important role of cyclin C (CCNC) in regulating human HSPC quiescence, as knocking down CCNC expression in human cord blood (CB) CD34+ cells resulted in a significant increase in quiescent cells that maintain CD34 expression. CCNC knockdown also promotes *in vitro* HSPC expansion and enhances their engraftment potential in sub-lethally irradiated immunodeficient mice. Our studies establish cyclin C as a critical regulator of the G_0/G_1 transition of human HSPCs and suggest that modulating cyclin C levels may be useful for HSC expansion and more efficient engraftment.

Keywords

cyclin C; hematopoietic stem/progenitor cell; human cord blood CD34+ cells; quiescence; engraftment

[*]Correspondence to: Stephen D. Nimer. Molecular Pharmacology and Chemistry Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065, USA., Phone: (646) 888-3040, Fax: (646) 422-0246,, s-nimer@mskcc.org.

Author Contributions:
Yasuhiko Miyata: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, manuscript writing
Yan Liu: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, manuscript writing
Vladimir Jankovic: Collection and/or assembly of data, Data analysis and interpretation, manuscript writing
Goro Sashida: Provision of study material or patients
Jennifer May Lee: Collection and/or assembly of data
Jae-Hung Shieh: Provision of study material or patients
Tomoki Naoe: Provision of study material or patients
Malcolm Moore: Conception and design, Data analysis and interpretation
Stephen D. Nimer: Conception and design, financial support, administrative support, data analysis and interpretation, manuscript writing, final approval of manuscript.

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Introduction

Hematopoietic stem cells (HSCs) play an essential role in the maintenance of multi-lineage blood production throughout the life of the organism by their dual abilities to self-renew and to differentiate into progenitors of various lineages [1]. Quiescence is an important property of HSCs, which is critical for maintaining hematopoietic proliferative capacity throughout life. The relative quiescence of HSCs also protects them from DNA damage caused by oxidative stress, chemotherapy or irradiation [2–4].

A number of genes and signaling pathways have been implicated in regulating HSC quiescence [5]. Several cell cycle regulators have been shown to play critical roles in HSC and/or HPC proliferation, including p21, p27, p57, p16, p18, as well as the D-type cyclins and their catalytic partners Cdk4 and Cdk6 [6–13]. HSC cell fate decisions are also regulated by several transcription factors (Gfi-1, Pbx-1, MEF/ELF4, C-myc and N-myc) [14–17]. Interestingly, recent studies indicate that tumor-suppressor genes, including PTEN, p53, Rb, PML, APC and Fbw7, may play critical roles in maintaining HSCs in a quiescent state [18–23]. Besides HSC intrinsic mechanisms, HSC function is also regulated by ligand-receptor interactions, including those between angiopoietin and Tie-2, thrombopoietin (TPO) and c-Mpl, stem cell factor (SCF) and c-Kit, and stromal-derived factor 1 (SDF-1) and CXCR4 [2, 24–26].

While most of our understanding of HSC quiescence comes from studies of murine hematopoiesis, little is known about the regulation of human HSC quiescence. In human CB CD34+ cells, interrupting p21 expression *ex vivo* resulted in expanded stem cell number and enhanced *in vivo* stem cell function compared with control, manipulated cells [27]. We have shown that MEF/ELF4, an ETS transcription factor, regulates murine HSC quiescence and that acute knock down of MEF in human CB CD34+ cells also enhances HSPC quiescence [16]. In contrast, quiescent human CB HSPCs express more GATA-2 than cycling HSPCs and inducing GATA-2 expression increases quiescence [28].

HSC quiescence is maintained by the balance between positive and negative proliferative factors: Both stromal-derived factor 1 (SDF-1) and transforming growth factor β (TGFβ) have been found to inhibit the cycling of primitive human progenitor cells *in vitro* [9, 29], and treatment of NOD/SCID mice with either SDF-1 or TGFβ *in vivo* significantly enhances the transplantability of human HSPCs [32–33]. In general, quiescent HSCs have been shown to exhibit higher repopulating ability than proliferating HSCs in bone marrow transplantation assays [30–31].

In contrast to the well-characterized cyclin/cdk-mediated sequential inactivation of the retinoblastoma protein (Rb), that controls the G_1/S transition [34], little is known about the regulation of the G_0/G_1 transition. One potential regulator of this process is cyclin C (CCNC), which was first isolated as a human cDNA that could substitute for the G_1-cyclin genes in *S. cerevisiae* [35–36]. Cyclin C and Cdk3 have been shown to regulate the exit of human fibroblasts from the quiescent state into the G_1 phase [37] and because CCNC expression is up-regulated during the exit of murine LT-HSCs from G_0 [38], we hypothesized that cyclin C could regulate the G_0/G_1 transition in human hematopoietic stem/progenitor cells. Using RNA interference to knock down CCNC expression in human CD34+ cells, we demonstrate that cyclin C plays an important role in regulating HSPC quiescence. Knockdown of CCNC in these cells increased quiescence, promoted HSPC expansion and enhanced their engraftability in sub-lethally irradiated immunodeficient mice. Therefore, cyclin C is an important regulator of the G_0/G_1 transition in human HSPCs.

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Materials & Methods

Cells and cell culture

Human cord blood (CB) cells from volunteer donors were obtained from the New York Blood Center (New York, NY) and Tokai Cord Blood Bank (Nagoya, Japan). CD34+ cells were purified as described previously [39], by density gradient centrifugation using Ficoll-Paque Plus and then positive selection using the Miltenyi MACS CD34 Isolation Kit (Miltenyi Biotec). Cytokine-driven liquid (Delta) cultures were performed in QBSF-60 serum-free media (Quality Biological) containing 10 ng/mL recombinant human TPO (rhTPO), recombinant human SCF (rhSCF) and recombinant human Flt3 ligand (rhFlt3L). 293T cells were grown in modified Eagle medium (MEM) supplemented with 10% FBS and 2 mM L-glutamine. MS-5 murine stroma cells were grown in alpha MEM (α-MEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin. MS-5 stromal co-culture and long-term culture-initiating cell (LTC-IC) experiments were performed in α-MEM containing 12.5 % FBS, 12.5 % horse serum and 2 mM glutamine, 40μg/ml gentamicin, 250 μg/ml amphotericin B and 1 μM hydrocortisone.

Lentiviral vectors and reagents

The lentiviral vector FG12 (which expresses GFP under the Ubiquitin C promoter and an shRNA under the human U6-RNA Pol III promoter) was kindly provided by David Baltimore [40]. To generate shRNA-expressing lentiviral vectors capable of targeting human cyclin C, cyclin D1, cyclin D2 or cyclin D3, oligonucleotides directed against cyclin C mRNA at nucleotides 468–486 and 582–600 (GenBank accession number gi: 61676090), cyclin D1 mRNA at nucleotides 5383–5401 (Gen Bank accession number gi: 166795258), cyclin D2 mRNA at nucleotides 1454–1472 (GenBank accession number gi: 16950656) and cyclin D3 mRNA at nucleotides 1585–1603 (GenBank accession number gi: 16950657) were subcloned into the FG12 vector (FG12 shCCNC, FG12 shCCNC2, FG12 shCCND1, FG12 shCCND2 and FG12 shCCND3, respectively). For the control shRNA, a shRNA against LacZ was used (FG12 shLacZ), with the target sequence being the 1915–1933 region of the bacterial galactosidase gene (LacZ; gtgaccagcgaatacctgt). Recombinant human SCF, interleukin (IL) -3, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and TPO were kindly provided by Kirin (Japan). Recombinant human granulocyte CSF (rhG-CSF) and erythropoietin (rhEpo) were obtained from Amgen, and Ortho Biotech, respectively. Recombinant human Flt3 ligand was purchased from Peprotech. An antibody against human cyclin C (sc-1061) was purchased from Santa Cruz. Fluorescence-conjugated antibodies to human CD34, CD44 and CXCR4 were purchased from BD Biosciences.

Lentiviral production and transduction into CD34+ cells

293T cells were used to produce lentiviruses by co-transfection of packaging plasmids and the FG12 lentiviral vector. Human CD34+ cells were cultured for 5 to 8 hours in the presence of rhTPO (10 ng/ml), rhSCF (10 ng/ml) and rhFlt3L (10 ng/ml) in QBSF-60 serum-free media, and then infected by spin-inoculation in the presence of 4 μg/ml of polybrene (Sigma), at 1300 rpm for 45 min two or three times with 12-hour intervals. Forty-eight hours following the last infection, GFP positive cells were purified by FACS and used for further experiments. For the transplantation experiments, only one round of transduction was performed to minimize the loss of stemness during the transduction period.

siRNA electroporation into CD34+ cells

A control non-silencing scrambled siRNA and an siRNA directed against human CCNC mRNA (siCCNC) were synthesized by the High-Throughput Drug Screening Facility at MSKCC (sequences available upon request). Nucleofection was performed using the

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Nucleofector™II kit (Amaxa), according to the manufacturer's directions. Briefly, 1×10^6 CD34+ cells were mixed with 1.5 μg of siRNA and electroporated, using the U-08 program.

RNA isolation and Quantitative PCR

RNA extraction was performed using the RNeasy Plus kit (Qiagen). cDNA was synthesized using the SuperScript™ III First-Strand Synthesis System for RT-PCR kit (Invitrogen), according to the manufacturer's instructions. Quantitative PCR was performed using an ABI 7500 instrument and analyzed using Sequence Detector Software (Applied Bioscience). The expression level for each gene was normalized to the level of hypoxanthine phosphoribosyltransferase (HPRT) expression. The PCR primer sequences used are available upon request.

Western blotting analysis

Cells were washed with phosphate-buffered saline (PBS), and lysed in lysis buffer (1% TritonX-100, 150 mM NaCl, 20 mM Tris (pH 8.0), 1 mM EDTA, and protease inhibitors). The protein lysates were briefly sonicated and centrifuged for 15 minutes at the maximum speed. The protein lysates were mixed with 4x SDS loading buffer, boiled for 5 minutes, and run on a 10 % NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA). The protein was transferred to a polyvinylidene fluoride membrane, and the membrane blocked in 5% non-fat dry milk in PBS. The blot was incubated with a primary antibody, washed with PBS containing 0.05% Tween-20, and incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham). The blot was developed using a chemiluminescent substrate and exposed on autoradiography film.

Hoechst 33342/Pyronin Y staining

Purified CD34+ cells were stained with 2.5 μM Hoechst 33342 (Molecular Probes) for 45 minutes at 37 °C. Pyronin Y was added at the final concentration of 1 μM and cells were further incubated for 45 minutes at 37 °C. Thereafter, cells were kept on ice for 30 minutes. Cells were washed 2 times and stained with APC-conjugated anti human CD34 antibody.

Ki-67 expression analysis

Forty-eight hours after nucleoporation, human CD34+ cells were collected, washed and resuspended in PBS containing 1% formaldehyde, and incubated for 30 minutes at 4 °C. An equal volume of PBS containing 0.2 % Triton X-100 was then added, and the cells were stored overnight at 4 °C. The cells were washed twice and incubated with FITC-conjugated anti-human Ki-67 monoclonal antibody (BD) for 30 minutes. The cells were then washed twice with PBS, resuspended and incubated at 4 °C in PBS containing 5 μg/ml 7-aminoactinomycin-D (7-AAD) for 3 hours.

Cell division tracking and Cell-cycle analysis

CD34+ cells were labeled with 2.5μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes) in PBS for 10 min at 37 °C, as described previously. After a variable period of time in liquid culture, the cells were analyzed for fluorescence intensities of CFSE using flow cytometry.

For propidium iodide (PI) staining, cells were centrifuged and fixed in 70 % ethanol for 2 hours. Cells were washed twice with PBS containing 2 % BSA and finally resuspended in PBS containing 2 % BSA, 140 μg/ml RNase A and 50 μg/ml PI.

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Colony-forming unit (CFU) assay

CFU assays were performed in semi-solid medium as previously described [39], with modifications. Briefly, 500 sorted cells were plated in 35-mm plates using 0.9 % methylcellulose containing 20 % BIT9500 (Stem Cell Technologies), 2 mM glutamine, 50 ng/mL rhSCF, 20 ng/mL rhIL-3, 20 ng/mL rhIL-6, 20 ng/mL rhG-CSF, 20 ng/mL rhGM-CSF and 6 U/mL rhEpo. Colonies were scored 14 days after plating. For secondary CFU assays, cells were harvested and washed with PBS on day 14 after the first plating and 5×10^4 cells were replated as described above.

The Delta assay is a short-term suspension culture based assay used to detect hematopoietic stem/primitive progenitor cells. Cells with colony-forming unit potential are first grown in liquid culture before they are replated in semi-solid medium. The numbers of colonies obtained are considered an indicator of the number of hematopoietic stem/progenitor cells present. For the Delta assay, 5×10^3 cells growing in liquid culture were plated on methylcellulose, and colonies were scored on day 14.

We performed CFU assays using bone marrow cells obtained from mice transplanted with human CD34+ cells, by plating 5×10^4 cells in methycellulose containing rhSCF and rhIL-3 (which do not cross-react with murine receptors) and rhEPO. Colonies were scored on day 14.

Cobblestone area–forming cell (CAFC) and long-term colony-initiating cell (LTC-IC) assays

CAFC assays were performed by plating CD34+ cells onto MS-5 stroma cells. Weekly demidepopulation was performed, with phenotypic analysis of the non-adherent cells. Cobblestone areas, defined as groups of at least 5 phase-contrast dark cells tightly associated beneath the MS-5 monolayer, were scored at week 5.

Limiting dilution LTC-IC assays were performed by plating sorted FG12 shLacZ and FG12 shCCNC transduced CD34+ cells in 96-well tissue-culture plates containing an MS-5 cell monolayer, at 6 progressive dilutions with 10 replicate wells per dilution. After 5-weeks of co-culture, the cells were harvested and plated in methylcellulose; CFUs were counted on day 14. The frequency of LTC-ICs was calculated using L-calc™ software (Stem Cell Technologies).

Transplantation into NOD/SCID IL-2Rγ null (NOG) mice

NOG mice were purchased from Jackson Laboratories and bred in the SKI core animal facility. Mice between 8 and 12 weeks old were irradiated with a single dose of 3.5 Gy. 1×10^5 transduced human CD34+ cells were injected into the mice via their tail vein. After 6 weeks, bone marrow cells were harvested and analyzed for human CD45 expression, to define the engraftment efficiency.

Statistical Analysis

Results obtained from multiple experiments are expressed as the mean ± standard deviation (SD), except as otherwise noted. The mean ± standard error of the mean (SEM) is used for describing the chimerism of human CD45+ cells in the transplanted NOG mice. The data were analyzed using a two-tailed unpaired Student's *t*-test. Probability values $< .05$ defined significant differences between test points

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Results

Up-regulation of CCNC expression during the G_0/G_1 transition of the quiescent human CB CD34+ cells

Given that CCNC (in cooperation with cdk3) mediates the G_0/G_1 transition in human fibroblasts [37], we investigated whether human CB CD34+ cells utilize a similar mechanism. We first examined the expression levels of CCNC in human CB CD34+ cells during various phase of the cell cycle. Magnetically purified CD34+ cells stained were sorted into $G_{0/1a}$, G_{1b} and $S/G_2/M$ fractions (Fig. 1A); to separate $G_{0/1a}$ cells from G_{1b} cells, we used Hoechst 33342 and Pyronin Y staining for DNA and RNA, respectively. In unstimulated cells, the level of CCNC mRNA did not fluctuate significantly during the progression of the cell cycle (Fig. 1B), whereas expression of the early G_1-cyclins, including cyclin D1 (CCND1), D2 (CCND2) and D3 (CCND3), were transiently up-regulated in the G_1 phase, consistent with their known role in G_1 progression [12, 41]. However, following cytokine stimulation, CCNC expression was up-regulated in the $G_{0/1a}$ cells, but not in the G_{1b} cells, suggesting a specific role of CCNC in regulating the G_0 to G_1 transition (Fig. 1C).

Knock down of CCNC expression in human HSPCs increases their quiescence

To determine whether knockdown of CCNC expression alters the behavior of HSPCs, we transiently transfected either a control siRNA or an siRNA directed against CCNC mRNA (siCCNC) into purified CD34+ cells using nucleoporation. CCNC mRNA expression decreased by 50% in siCCNC-transfected cells at 48 hours compared to the control siRNA-transfected cells (Fig. 2A). Using the Ki-67 proliferation marker, we found that the quiescent (Ki-67 negative) fraction increased in the CCNC knockdown cells, compared to the control cells (11.4% vs 7.8%, Fig. 2B). Moreover, using CFSE staining to monitor cell divisions, we found a much higher frequency of cells within the siCCNC transfected CD34+CD38- cells that had not divided by 48 hours post transfection, compared to the control siRNA transfected cells (Fig. 2C lower panel, $p = 0.0002$, $n = 4$). This suggests that transient down-regulation of CCNC in human HSPCs is sufficient to increase their quiescence.

We then used a lentiviral-based system that can efficiently targets non-dividing cells to express shRNA constructs in primary human CD34+ cells (including those in the G_0 phase of the cell cycle). Magnetically purified human CB CD34+ cells were transduced with the FG12-shCCNC lentiviruses or the control FG12-shLacZ lentiviruses. The transduction efficiencies were similar, as assessed by GFP expression which was positive in 50–80% of the cells; the infected cells remained GFP positive for 5-weeks in liquid culture (Fig. 3A). The FG12 shCCNC lentiviruses significantly decreased CCNC RNA and protein expression (at 48 hours post-transduction) as assayed by both quantitative PCR and immunoblotting assays (Fig. 3B). We then assessed the proportion of $G_{0/1a}$ cells (48 hours post-transduction) using Hoechst 33342 and Pyronin Y staining. As shown in Figure 3C, CCNC knockdown significantly increased the proportion of CD34+ cells in the $G_{0/1a}$ phase (4.3 ± 0.8 % vs. 1.5 ± 0.4 % $p = 0.002$), and this effect was specific to the $G_{0/1a}$ phase, as there was no difference in the $G_1/S/G_2M$ profile of the cells 48 hours post-sorting or 7 days post-sorting (Fig. 3D). To rule out off-target effects of this shRNA, we tested another shRNA against CCNC (FG12 shCCNC2) that efficiently knocked down cyclin C levels (Fig S1A). We observed a similar increase in G_0/G_{1a} cells in CD34+ cells transduced with FG12shCCNC2 (Fig. S1B). This further demonstrates that CCNC is a critical regulator of human HSPC quiescence.

CCNC knockdown does not affect the differentiation of committed progenitors

To determine whether knockdown of CCNC expression affects myeloid differentiation, we performed standard methylcellulose colony forming unit (CFU) assays. We observed no

difference in total colony numbers (Fig 4A, 110 ± 8.9 vs. 124 ±6.1 colonies/500 cells n = 3 p = 0.088), lineage composition, or in the appearance of the colonies generated by the CCNC knockdown cells vs. the control cells. Similarly, CCNC knockdown did not affect the differentiation of cells growing on MS-5 stroma cells (where exogenous cytokines are not present) (see flow cytometry data in Fig. S2). Thus, CCNC does not appear to play a critical role in the differentiation of committed progenitor cells. Interestingly, CCNC knockdown cells growing in liquid culture generated more CFUs than the shLacZ transduced cells at weeks 2 and 3, as measured by the delta assay (Fig. 4B). Similarly, knockdown of CCNC in the CD34+CD38- cells also led to a significant increase in the frequency and number of CFUs (Fig. 4C, p = 0.0005, n = 3). Thus, cells in which CCNC is knocked down retain primitiveness, leading to a greater capacity to generate CFUs.

CCNC knockdown maintains the immature cell surface markers and morphological features of human HSPCs *in vitro*

To further define how lowering CCNC levels affects the primitiveness of human HSPCs, we examined the proliferative capacity of transduced CD34+ cells in early-acting cytokine (rhSCF, rhTPO and rhFlt-3L) containing liquid cultures. The initial expansion of CCNC knockdown cells was slower compared to control transduced cells during the first two weeks of culture, likely due to a slower rate of cell cycle entry (Fig. 5A). [A similar effect was observed in CCNC knockdown human HSPCs when they were co-cultured with MS-5 stroma cells (under non-cytokine-driven conditions; Fig. S3)]. Yet, the cumulative expansion of the CCNC knockdown cells over 5 weeks in culture was significantly greater than the shLacZ transduced cells (1840 fold vs. 830 fold, n=4, p=0.028, Fig. 5B).

We also quantified the CD34 expression of transduced cells over time:While the initial frequency of CD34+ cells was similar, a significantly greater proportion of the CCNC knockdown CD34+ cells retained CD34 expression over 4-weeks in culture (Fig. 5C), with a higher frequency of CD34+ CD38−/low cells, compared to the control transduced cells (Fig. 5D). To further investigate this property, CD34+ cells were separated into the CD34+CD38− (HSCs) and the CD34+CD38+ (committed progenitors) fractions, followed by transduction with either the FG12 shLacZ or FG12 shCCNC vectors. As shown in Fig. 5E, a significant proportion of the CCNC knockdown CD34+CD38− cells retained CD34 expression (with an immature morphologic appearance, Figure 5F), while nearly all of the control transduced CD34+CD38− cells became CD34 negative (Fig. 5E). Similar to the results seen in stroma-free cultures, CCNC knockdown led to a delayed expansion of GFP+ (non-adherent) cells (Fig. 5G). CCNC knockdown had no significant effect on HSPC apoptosis, as measured by Annexin V staining (Fig. S4), suggesting that the maintenance of CD34+ cells is not due to decreased apoptosis. Rather, the maintenance of CD34+ cells with CCNC knockdown likely reflects preservation of their in vitro self-renewal capacity, due to enhanced quiescence.

Reducing CCNC expression enhances the expansion HSPC and enhances its engraftment in immunodeficient mice

We next performed cobblestone area-forming cell (CAFC) assays to assess the effect of lowering CCNC levels on HSPC self-renewal. To ensure that the initial cell populations contained similar numbers of quiescent cells, equal numbers of cells that remained CFSE bright 3 days after electroporation were co-cultured on MS-5 stromal cells. We found a 3-fold increase in CAFCs in the siCCNC-transfected cells, compared to the control siRNA transfected cells (Fig. 6A, 3.4 ± 0.15 fold, p = 0.0014, n = 4). Similarly, shRNA mediated CCNC knockdown in human CB CD34+ cells also resulted in a greater CAFC frequency (49 cells vs. 21 cells/5×10^4 cells plated; n=4, p<0.05; Fig. 6B). Moreover, CCNC knockdown CD34+CD38− cells generated 3.6-fold more CAFCs than the control shRNA

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transduced cells; virtually no CAFCs were detected in either of the transduced CD34+CD38+ cells (Fig. 6C 5.4 ± 2.1 cells vs. 1.5 ± 0.7 cells/5 × 10^4 cells plated, *p = 0.015, n = 4).

Quiescent HSCs exhibit a significantly higher engraftment potential than proliferating HSCs in bone marrow transplantation assays [30–32]. Therefore, we sought to determine whether the enhanced quiescence seen in CCNC knockdown CD34+ cells would improve their engraftability in immunodeficient mice. Lentiviral shRNA-transduced CD34+ cells were injected intravenously into NOD/SCID IL-2Rγ null (NOG) mice after sublethal irradiation, and chimerism was analyzed 6 weeks later, by monitoring human CD45 expression in the cells isolated from mouse bone marrow. We observed a significant enhancement of CCNC knockdown cell engraftment, compared to the control shRNA transduced cells (Fig. 6D), despite similar transduction efficiencies (based on percent GFP positivity; Fig. S5). Furthermore, we also found a significant increase in the numbers of human CFUs in the marrow of the mice that received CCNC knockdown cells (Fig. 6E, 101.4 ± 42.7 vs. 2.3 ±1.9 colonies/50,000 cells; $p < 0.01$, n = 3). Thus, CCNC appears to restrict the self-renewal capacity of HSPCs by promoting their cell cycle entry, and knockdown of CCNC leads to enhanced quiescence and better engraftability. While CXCR4 and CD44 are involved in HSC homing to the bone marrow [29], we observed no change in their expression in CCNC knockdown cells (Fig. S6), suggesting that the enhanced engraftment of CCNC knockdown cells in immunodeficient mice is due to their increased quiescence, and not due to the altered expression of homing receptors. Furthermore, we performed homing assays and found no difference in the homing ability of CCNC knockdown cells vs control knockdown cells (data not shown).

Discussion

A variety of cell cycle regulatory proteins, transcription factors and cell signaling molecules have been shown to regulate the quiescence of murine hematopoietic primitive stem/ progenitor cells [2–26], using genetically modified mice and bone marrow transplantation studies. While identification of genes that control the quiescence of human primitive progenitor cells has proved to be difficult, we have taken advantage of RNAi, improved primary cell transduction protocols, and xenotransplant assays, to define the role of CCNC in regulating the G_0/G_1 transition in human HSPCs (CB CD34+ cells). We found that CCNC mRNA is up-regulated during cytokine stimulation of the G_0/G_1 transition of human CB CD34+ cells and that reducing CCNC levels by siRNAs or shRNAs directed against CCNCC increases the quiescent population of human CD34+ cells. While CCNC knockdown had onlya minimal effect on CD34+CD38+ committed progenitors, its knockdown in CD34+CD38− cells led to retention of CD34+ expressing cells and maintenance of colony-forming ability after long-term culture. This finding, coupled with no apparent effect on the differentiation potential of the CD34+ cells, shows that it is possible to manipulate the cell cycle of HSPCs without significantly altering the differentiation program.

D-type cyclins (cyclin D1, D2, and D3) were thought to play a global role in cell cycle progression, yet when Sicinsky and colleagues generated mice lacking all D-type cyclins, they found that D-type cyclins were primarily required for HSC expansion [12]. Similarly, Barbacid and colleagues showed that Cdk4 and Cdk6 seem essential for HSC proliferation, but not for proliferation in other tissues [13]. CyclinD3 is highly expressed in long-term HSCs [41], further highlighting the important roles that CyclinD/Cdk4/6 complexes could play in the regulation of HSC proliferation. The human CCNC gene was originally isolated based on its ability to rescue G_1-cyclin deficient yeast [35–36]. Given the potential redundancy of cyclin C with D type cyclins, we examined whether knockdown of the early

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G_1-cyclins, CCND1, CCND2 and CCND3, could also affect HSPC immaturity. Knockdown of CCND1 or CCND3 had no effect on CD34 maintenance, while knockdown of CCND2 caused a modest increase in CD34+ cells in liquid culture at week 2 which was much less than that seen using CCNC knockdown cells (Fig. S7). Thus, it appears that CCNC has a relatively unique role in maintaining the immaturity of human CD34+ cells.

Several recent studies have shown that quiescent HSCs exhibit higher engraftment potential when transplanted into lethally irradiated recipient mice [30–32]. We also observed higher chimerism in immunodeficient mice transplanted with CCNC knockdown cells than that seen using shLacZ transduced human CD34+ cells, indicating that down-regulating CCNC expression can improve the engraftability of human CD34+ cells. The improved engraftment of CCNC knockdown CD34+ cells further supports the hypothesis that CCNC plays an important role in primitive progenitors (CD34+CD38− cells), rather than the more committed progenitor cell compartment (CD34+CD38+ cells). This relationship between quiescence and engraftability is not always seen: it appear that lowering p21 levels in human CB CD34+ cells (thereby decreasing quiescence) enhances the engraftment of CB CD34+ cells in immunodeficient mice, whereas cells with the highest levels of exogenous GATA-2 expression (which are more quiescence) fail to contribute to hematopoiesis in NOD-SCID mice [27–28].

The pRb protein, a member of the family of “pocket proteins” that also includes p107 and p130, plays an important role in regulating the G_1 checkpoint, cellular differentiation, apoptotic cell death, permanent cell cycle arrest and chromosomal stability [34]. pRb is likely to participate in the regulation of quiescence, because its acute somatic inactivation is sufficient for G_0-arrested cells to reenter the cell cycle [42]. Similarly, formation of p130/E2F4 complexes is thought to be a characteristic of G_0 and during the transition of cells from G_1 to G_0, p130 undergoes a specific phosphorylation event leading to its association with E2F4 [43]. While the conditional deletion of Rb does not alter hematopoiesis [44], loss of all three Rb family members in the HSC compartment leads to enhanced HSC proliferation, suggesting that HSC quiescence can be maintained by the presence of any one of the retinoblastoma family of proteins [20]. Rollins and colleagues showed that cyclinC/Cdk3 phosphorylated pRb at Serine807/811 during the G_0/G_1 transition of human fibroblasts and that this phosphorylation is required for cells to exit G_0 efficiently [37, 43]. A similar mechanism may occur in human HSPCs.

The ability to increase the number of human HSCs *in vivo* or *in vitro* could provide improved treatment options for clinical transplantation and gene therapy protocols, however clinically relevant expansion of HSCs has been difficult to achieve [45]. Understanding the molecular mechanisms regulating human HSC quiescence and self-renewal may be useful for improving the engraftability of human HSCs and may also facilitate the development of therapeutic strategies that can eliminate the largely quiescent leukemia (or cancer) stem cell. Our work suggests that regulating the expression or activity of CCNC in human HSCs may provide a useful therapeutic strategy to enhance engraftability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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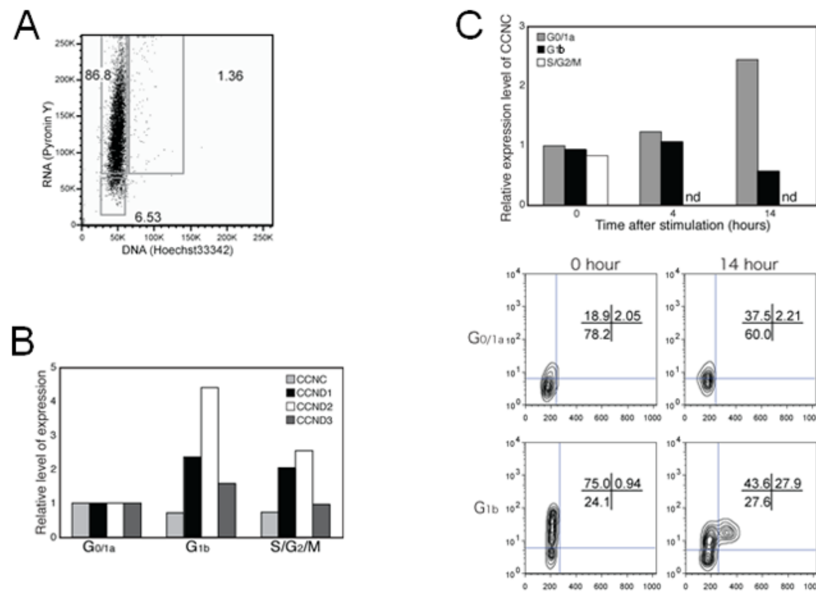

Figure 1. Cyclin C (CCNC) expression in human CB CD34+ cells

A. The quiescence of human CB CD34+ mononuclear cells was analyzed by using Hoechst 33342 and Pyronin Y staining. The left lower gate, the left upper gate and the right gate represent the G_0 fraction, the G_1 fraction and the $S/G_2/M$ fraction, respectively.

B. Levels of CCNC, CCND1, CCND2 and CCND3 mRNA were measured by quantitative PCR in the cell populations shown in Fig. 1A. The expression level of each gene in the G_0 cells was set to 1 after normalization to HPRT expression. The values shown are an average from two experiments.

C. The cell fractions shown in Fig. 1A were stimulated with cytokines for 4 or 14 hours, and CCNC mRNA levels were measured. The value of the unstimulated G_0 cells was set to 1, after normalization to HPRT expression. The values shown are averages from two experiments. nd = not done. The cell cycle profile of CD34+ $G_{0/1a}$ and G_{1b} cells is shown at time 0 and after 14 hours in culture (flow plots in bottom half of figure).

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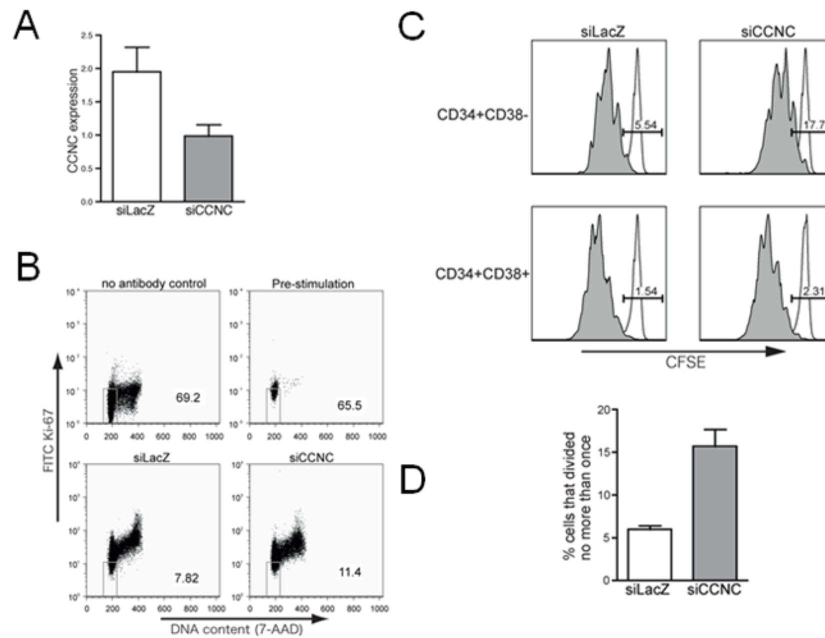

Figure 2. Transient knockdown of CCNC is sufficient to increase HSC quiescence

One million CD34+ cells were nucleoporated with 1.5 μg of either a control siRNA or a siRNA directed against CCNC mRNA (siCCNC) and then cultured in serum-free medium containing cytokines.

A. CCNC expression levels were measured 48 hours after nucleoporation by quantitative PCR, and normalized to HPRT expression (n=3).

B. Transiently transduced CD34+ cells were fixed and permeabilized 48 hours after nucleoporation, and then stained with Ki-67 and 7-AAD to assess quiescence. The upper right panel shows the cell cycle status of the unstimulated CD34+ cells. The lower left and right panels show the cell cycle status of CD34+ cells nucleoporated with a control siRNA or the CCNC directed siRNA, respectively.

C. CD34+ cells were stained with CFSE, transduced with siRNAs and then placed in liquid culture. The frequencies of CFSE "high" cells, that had not divided more than once, based on the CFSE intensity (the unfilled histogram), among the CD34+CD38−and CD34+CD38+ cells were analyzed 6 days post-nucleoporation by flow cytometry ($p = 0.0002$, $n = 4$).

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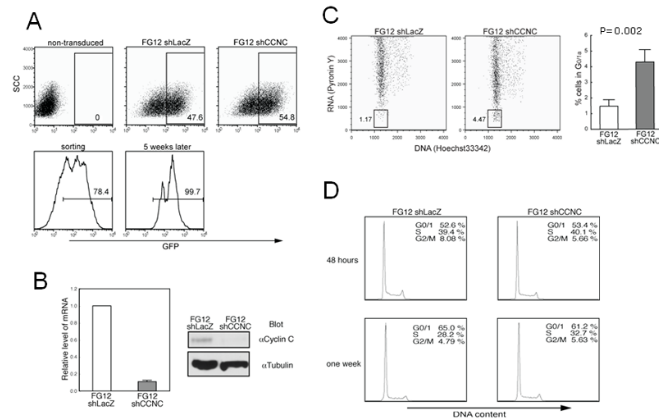

Figure 3. Down-regulation of CCNC increases the fraction of G_0 human CD34+ cells

A. Transduction efficiencies were determined by monitoring GFP expression using flow cytometry 48 hours after the last transduction. Purified CD34+ cells were transduced with either the control FG12 shLacZ lentivirus (upper middle panel) or FG12 shCCNC (upper right panel). The GFP bright population (gated in the lower left panel) was sorted and used for the *in vitro* assays. Most of the cells remain GFP positive 5-weeks later (lower right panel).

B. The efficient knockdown of CCNC at both mRNA and protein levels was confirmed by quantitative PCR (on the left) and immunoblotting (on the right) 48 hours after the last transduction. The level of CCNC mRNA is shown relative to HPRT expression. An anti-tubulin antibody was used to show equal protein loading in both lanes.

C. Flow cytometric analysis of the $G_{0/1}$ population of transduced CD34+ cells 48 hours after the last transduction, using cellular RNA staining (with Pyronin Y) and DNA staining (with Hoechst 33342). On the right is a graph showing the results of four independent experiments ($p = 0.002$).

D. Cell cycle analysis of FG12 shLacZ or FG12 shCCNC transduced CD34+ cells at the time of sorting (48 hours post-transduction, on the top) and 7 days post-sorting (on the bottom).

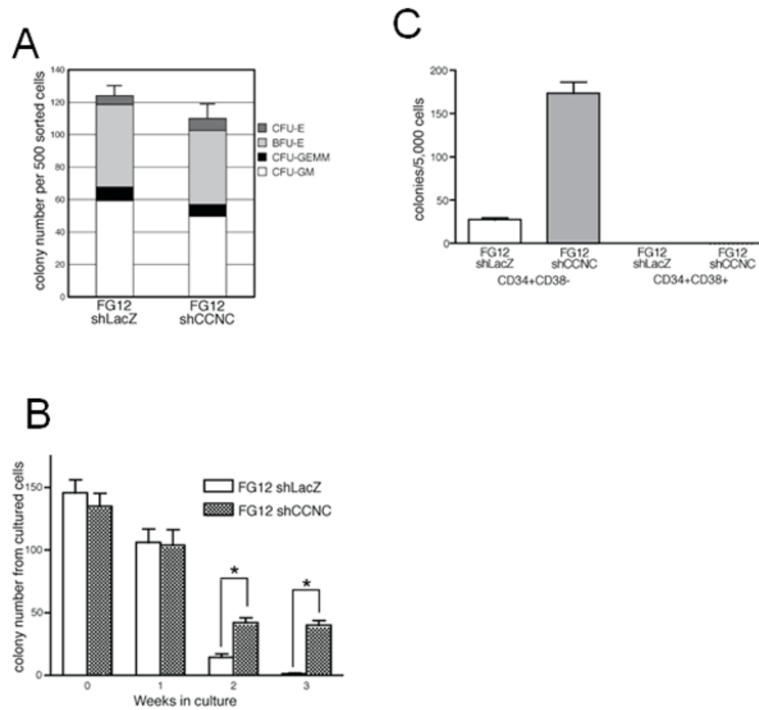

Figure 4. Knockdown of CCNC does not perturb the differentiation potential of committed progenitor cells

A. The CFC content of 500 transduced CD34+ cells was assayed in methylcellulose cultures, and the frequencies of BFU-E, CFU-E, CFU-GM and CFU-GEMM assessed using the appropriate cytokine combinations ($p = 0.088$, $n = 3$).

B. Preservation of CFU activity by CCNC knockdown in liquid culture (Delta assay). CFU assays were performed weekly using 500 cells at week 0 and 5,000 cells obtained from ongoing liquid cultures at subsequent weeks (*$p < 0.01$, $n= 3$).

C. Freshly isolated CD34+ cells were further sorted into CD38− and CD38+ fractions and transduced with either FG12 hLacZ or shCCNC before being cultured under various conditions. Preservation of CFC activity in CCNC knockdown CD34+CD38−cells, but not CD34+ CD38+ cells, at week 5 of the Delta assay. The CFC frequencies are shown (*$p = 0.0005$, $n=3$).

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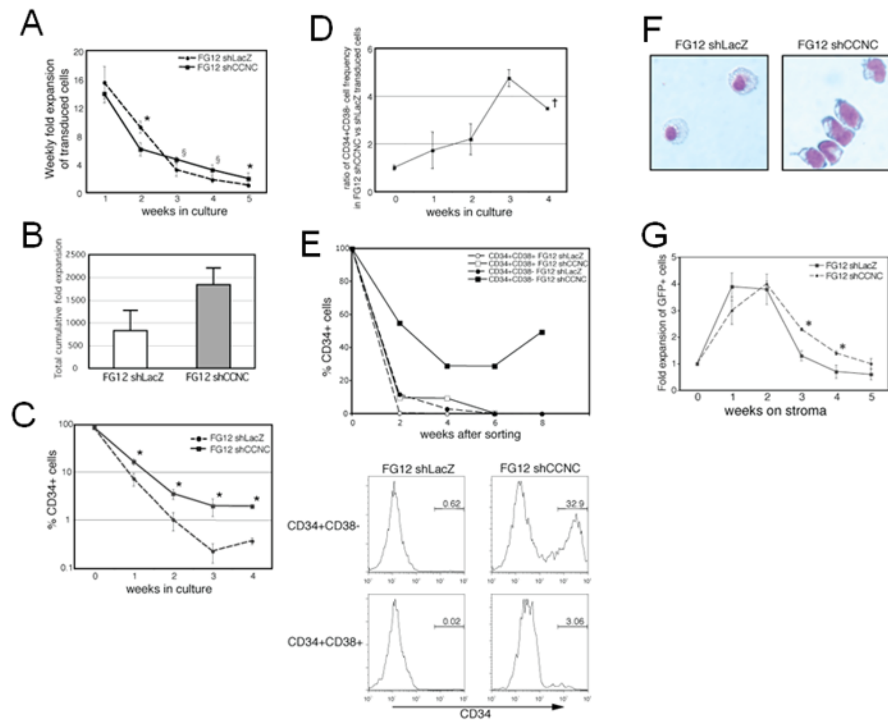

Figure 5. CCNC knockdown maintains the immature cell surface markers and morphological features of human CD34+ HSPCs *in vitro*

A, B. Transduced CD34+ cells (GFP+) were cultured in serum-free medium with cytokines, counted weekly and replated at 5×10^4 cells/ml. Weekly fold expansion (A) (* p < 0.01, § p < 0.05, n=4) and cumulative fold expansion (B) (p = 0.028, n = 4) of the transduced cells are shown for a 5-week period.

C, D. Persistence of CD34+ (C) and CD34+CD38− cells (D) over a 4-week culture period following the expression of shRNA directed against CCNC. The frequency of CD34+ CD38− cells is shown as the ratio between the CCNC knockdown cells and the control FG 12 shLacZ vector transduced cells (D) (* p < 0.01, n=4, † indicates an average of two experiments).

E. Preservation of CD34 expressing cells over time (on the top) starting with CD34+CD38− or CD34+CD38+ cells. Flow cytometric profiles of CD34 expression at week 5 are shown (on the bottom), following knockdown of CCNC in the CD34+CD38− cells and CD34+CD38+ cells.

F. FG12 shCCNC transduced CD34+CD38− cells possess more immature morphologic appearances, with basophilic cytoplasm and a higher nucleus/cytoplasm ratio at week 5 in culture.

G. CD34+ cells transduced with either FG12 shLacZ or FG12 shCCNC were plated on MS-5 stroma cells, and the fold expansion of the GFP expressing cells was assessed weekly for 5 weeks (* p < 0.01, n=3).

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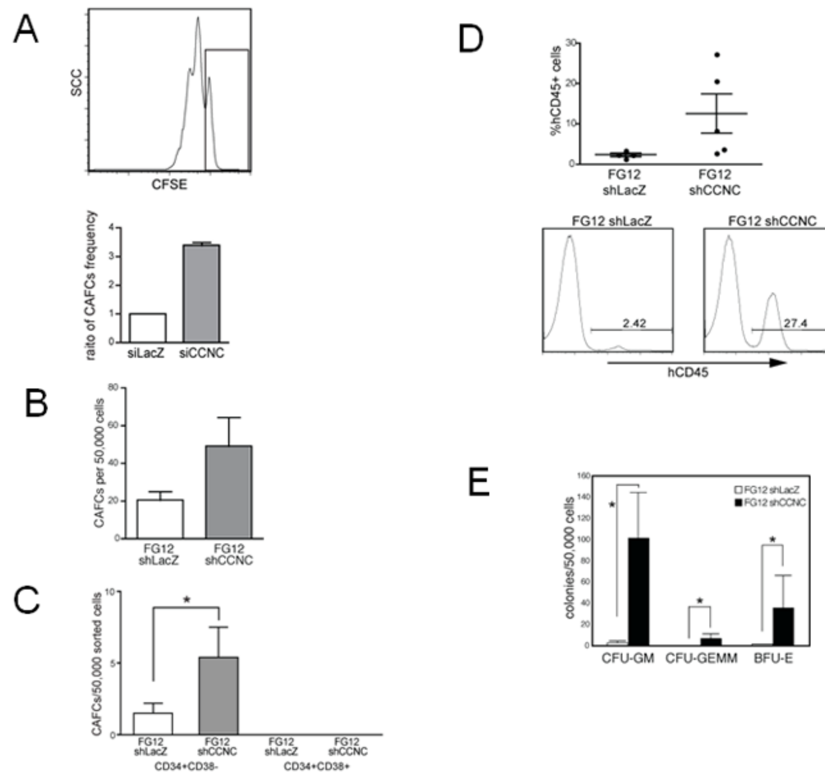

Figure 6. Reducing CCNC expression enhances the expansion of human HSPC and enhances its engraftment in immunodificient mice

A. CD34+ cells were stained with CFSE, transduced with siRNAs and then cultured in liquid medium. The frequencies of CFSE "high" cells that had not divided more than once in CD34+CD38− cells were analyzed 6 days post-nucleoporation by flow cytometry ($p = 0.0002$, $n = 4$; upper panel). Three days after nucleoporation, cells that had not divided were isolated (the box of the top panel) and 8000 sorted cells were plated on MS-5 stroma cells. The relative frequencies of CAFCs were then determined ($p = 0.00014$, $n = 4$; lower panel).

B. CAFC frequencies were quantified for the sorted FG12 shLacZ and FG12 shCCNC transduced human CD34+ cells at week 5 ($p < 0.01$, n=4).

C. CAFC assays were performed using transduced CD34+CD38− and CD34+CD38+ cells. The frequencies of CAFCs were assessed at week 5 ($p = 0.015$, $n = 4$).

D. Chimeric mice were analyzed based on the percentage of human CD45 expressing cells in their bone marrow. The mean and SEM from two independent experiments are shown in the upper panel, whereas representative profiles are shown in the lower panel.

E. The numbers of human CFUs per 50,000 bone marrow cells were assayed on methylcellulose, using a combination of rhSCF, rhIL-3 and rhEpo. Colonies were counted on day 14 for both the FG12 shLacZ and FG12 shRNA CCNC transduced cells (*$p < 0.001$, $n = 3$).