Generation of Dyskeratosis Congenita-like Hematopoietic Stem Cells through the Stable Inhibition of *DKC1*

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Short report

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Abstract

Dyskeratosis congenita (DC) is a rare telomere biology disorder, which results in different clinical manifestations, including severe bone marrow failure. To date, the only curative treatment for bone marrow failure in DC patients is allogeneic hematopoietic stem cell transplantation. However due to the toxicity associated to this treatment, improved therapies are recommended for DC patients. Here we aimed at generating DC-like human hematopoietic stem cells in which the efficacy of innovative therapies could be investigated. Because X-linked DC is the most frequent form of the disease and is associated with an impaired expression of DKC1, we have generated DC-like hematopoietic stem cells based on the stable knock-down of DKC1 in human CD34 + cells with lentiviral vectors encoding for DKC1 short hairpin RNAs. At a molecular level, DKC1 -interfered CD34 + cells showed a decreased expression of TERC, as well as a diminished telomerase activity and increased DNA damage, cell senescence and apoptosis. Moreover, DKC1 -interfered human CD34 + cells showed defective clonogenic ability and were incapable of repopulating the hematopoiesis of immunodeficient NSG mice. The development of DC-like hematopoietic stem cells will facilitate the understanding of the molecular and cellular basis of this inherited bone marrow failure syndrome, and will serve as a platform to evaluate the efficacy of new hematopoietic therapies for DC.

Introduction

Telomeres are repetitive nucleotide sequences localized at the end of the eukaryotic chromosomes, which play an essential role in the chromosome replication and stability. Telomeric DNA consists of tandemly repeated TTAGGG sequences (1, 2) which become shortened as a consequence of the division of somatic cells, leading to a situation called “end replication problem”. The loss of telomeric repeats is counteracted by the telomerase complex (3). Telomerase is a specialized ribonucleoprotein reverse transcriptase mainly composed of TERT (with reverse transcriptase activity), TERC (the RNA template) and dyskerin, which stabilizes telomerase complex (4-6). Although telomerase expression is low or absent in most somatic cells, telomerase remains active in somatic stem cells to maintain their telomere length (7). A decreased telomerase activity results in an abnormal telomere biology, leading to telomere biology disorders (TBD), such as aplastic anemia, pulmonary fibrosis, Coats plus syndrome or dyskeratosis congenita (DC) (2, 8).

Clinically, DC patients are characterized by the mucocutaneous triad (nail dystrophy, oral leukoplakia and abnormal skin pigmentation). Nevertheless, bone marrow failure (BMF) is the main cause of early mortality of these patients (80% of the cases) as also occurs in other congenic BMF syndromes (7). So far, 14 DC associated genes have been discovered, all of them involved in the telomere maintenance: *DKC1, TERT, TERC, TINF2*, *TCAB1*, *NOP10*, *NHP2*, *CTC1, RTEL1*, *TPP1*, *PARN*, *POT1*, *NAF1* and *STN1* (9-12). According to the inheritance of the disease, three DC variants have been reported: X-linked recessive, autosomal dominant and autosomal recessive. The X-linked variant of DC (X-DC) is mainly caused by point mutations in *DKC1,* which encodes for the dyskerin nucleolar protein (13). Interestingly, the knock-out of *Dkc1* has been reported to be embryonic lethal in mice (14). This observation and the fact that only

hypomorphic *DKC1* mutations have been reported in X-DC patients, (15, 16) reveals the critical relevance of *DKC1* in the cell biology.

To date, the only curative treatment for BMF in DC patients is the allogeneic hematopoietic stem cell transplantation (alloHSCT) from healthy donors. Apart from the low availability of HLA-matched donors, the outcome of DC patients undergoing alloHSCT is very poor, mainly due to the toxicity of conditioning regimens and the development of graft versus host disease (17). Thus, new therapies such as gene therapy without cytotoxic conditioning, as recently reported in Fanconi anemia (FA) (18), would be highly beneficial for DC patients.

Taking into account that periodic BM aspirations are not part of the routine follow-up of DC patients, difficulties in the access of HSCs constitute an important limitation in the development of new therapies for DC patients. Furthermore, the animal models of telomeropathies developed to date do not mimic the characteristic BMF of DC patients (19). Considering that *DKC1* is one the most frequently mutated genes in DC (9), the purpose of this study was the generation of DC-like human HSCs based on the interference of *DKC1* in human HSCs which would serve as a platform for the development of new hematopoietic therapies for DC patients.

Materials And Methods

Detailed methods are shown as Supplementary Data.

Results

Molecular implications of DKC1 inhibition in human hematopoietic stem and progenitor cells

Previous studies revealed that the knock-out of *Dkc1* is embryonic lethal (14) and that only hypomorphic mutations have been found in X-DC patients (15, 16). In this study we aimed at generating X-DC-like hematopoietic stem and progenitor cells (HSPCs) based on the down-regulation of *DKC1* with short hairpin RNA (shRNA) lentiviral vectors (LVs). shRNA-LVs carried a puromycin resistance gene to facilitate the selection of transduced HSPCs (see Materials and Methods).

The efficacy of seven different shRNA-LVs (**Suppl. Table 1**) to down-regulate the expression of *DKC1* was screened in healthy donor $CD34^+$ cells (**Suppl. Figure 1A**). In subsequent experiments, we showed that three of these shRNA-LVs, iDKC1, iDKC4 and iDKC7, significantly decreased *DKC1* mRNA levels to 34-47% compared to levels determined in cells transduced with the scrambled shRNA LV (**Figure 1A** and **Suppl. Table 2A**). Vector copy numbers (VCN) determined in these cells showed the presence of 1-8 copies per cell in all groups (**Suppl. Figure 1B**), revealing that inhibitory effects upon *DKC1* were related to the interfering proviruses.

To investigate the molecular implications resulting from the inhibition of *DKC1*, we first evaluated the expression of *TERC* in $CD34^+$ cells transduced with scrambled and *DKC1*-shRNA LVs. As shown in **Figure**

1B and **Suppl. Table 2B**, *TERC* mRNA levels in cells transduced with iDKC1-, iDKC4- or iDKC7-LVs were respectively decreased to 27.7%±10.8%, 49.1%±18.6% and 19.8%±11.5%, compared to levels determined in the control group. In subsequent analyses, changes in the telomerase functionality of *DKC1*-interfered $CD34^+$ cells were quantified. To this end, we measured telomerase activity of *DKC1*-interfered and control $CD34^+$ cells by the TRAP assay. These results showed marked decreases in the telomerase activity of $CD34^+$ cells that had been transduced with iDKC1-, iDKC4- or iDKC7-LVs, which showed values of 42.8% ±19%, 61.5%±3.6% and 43.7%±15.6%, respectively, of values determined in the control group (**Figures 1C** and **Figure 1D**).

In the following experiments we investigated the implication of *DKC1* interference in the DNA damage determined in $CD34^+$ cells. Analyses of γH2AX foci in the nucleus of cells transduced with iDKC1-, iDKC4- or iDKC7-LVs revealed that only 19% of cells transduced with the scrambled shRNA LV showed more than 10 γH2AX foci per cell. However, an important increase in the proportion of $CD34^+$ cells with γH2AX foci was observed in cells transduced with either the iDKC1- (76%), iDKC4- (42%) or the iDKC7- (61%) LVs (**Figure 2A**). In next studies, we determined the expression of phosphorylated p53 and p21 (*CDKN1A*) in $CD34^+$ cells transduced with the different constructs. As shown in **Figure 2B**, phosphorylated p53 expression was higher in $CD34^+$ cells transduced with the *DKC1*-shRNA LVs. When the expression of p21 was tested, iDKC1- and iDKC4-LVs enhanced its levels (2.7±0.7 and 2.4±0.26 fold, respectively) compared to the control group, though this was not observed in iDKC7-transduced cells (**Figure 2C** and **Suppl. Table 2C**). Levels of caspase 3 and Annexin V^+ cells were also increased in $CD34^+$ cells transduced with either type of *DKC1*-shRNA LVs, although levels did not reach statistical significance (**Figures 2B and 2D** and **Suppl. Figure 2**). Taken together these results suggest the induction of DNA damage, cell senescence and apoptosis of *DKC1*-interfered HSPCs (**Figure 2**).

The interfered expression of DKC1 impairs the in vitro growth and ablates the in vivo repopulating ability of human HSPC

To determine whether the knock-down of *DKC1* affects the functionality of human HSPCs, *DKC1*-interfered $CD34^+$ cells were *in vitro* cultured for 10 days (see Materials and Methods) to evaluate implications in cell growth. In these studies, the portion of $CD34^+$ cells at the end of the culture period was similar among the different experimental groups (**Suppl. Figure 3)**. While transduced cells with the scrambled shRNA-LV showed a marked cell expansion during this period (117±87.31 fold compared to initial cell numbers), levels of expansion observed in iDKC1- and iDKC4-transduced $CD34^+$ cells were only 13±6.99 and 15.3±2.42 fold compared to input cell numbers (**Figure 3A**). These values represent a significant decrease to 20±8% and 10±4%, respectively, of cell expansions corresponding to the control group ($CD34^+$ cells transduced with the scrambled shRNA LV) (**Figure 3B**). As happened with p21 levels (**Figure 2C**), defects in cell proliferation were not observed with iDKC7-transduced cells (**Figure 3B**). In additional studies we evaluated changes in the telomere length in *DKC1*-interfered cells, although no differences were observed among the different experimental groups (**Suppl. Figure 4**). This suggests that much longer incubation periods would be required to observe a significant telomere shortening, although

defects in the ability of *DKC1*-interfered cells to grow in culture limited the possibility of evaluating changes in the telomere length long-term after *DKC1*-interference.

Discussion

The absence of good models which mimic HSC defects characteristic of DC patients (20) constitute an important limitation in the development of therapies for the treatment of BMF of these patients (21). In this study we show that three different *DKC1*-shRNAs inhibited *DKC1* expression to levels below 50%, similar to observations in X-DC patients, all of them with hypomorphic mutations in *DKC1* (22, 23). Consistent with data from these patients (22-25), *DKC1* inhibition in healthy HSPCs was associated with a significant reduction in the expression of *TERC* and of telomerase activity. As also observed in cells from DC patients, *DKC1* interference with iDKC1- and iDKC4-LVs induced markers of DNA damage, cell senescence and apoptosis, such as the generation of nuclear γH2AX foci and up-regulation of caspase 3, p21 and phosphorylated p53.

Consistent with observations showing that BM from DC patients contain reduced numbers of HSPCs (26), *DKC1* interference with iDKC1- and iDKC4-LVs markedly reduced the cell expansion, as well as the clonogenic and *in vivo* repopulating potential of $CD34^+$ cells. The fact that, in contrast to iDKC1- and iDKC4-, iDKC7-LV did not increase levels of p21 nor affected the cell growth of $CD34^+$ cells suggests the different functional implications associated with the interference of different domains of *DKC1*.

Remarkably, defects in the *in vitro* and *in vivo* growth of human HSPCs were evident immediately after *DKC1* interference, despite no changes in the telomere length of these cells were observed. This observation indicates that the inhibited proliferation and repopulation ability of DC-like HSPCs, and most probably of HSCs from X-DC patients, is not necessarily a consequence of the reduced telomere length. Thus, we propose that the generation of DNA damage and induction of cell senescence and apoptotic responses would account for these relevant phenotypic defects of DC HSPCs. Although the inability of *DKC1*-interfered HSCs to engraft in immunodeficient mice would limit studies of the behavior of these cells *in vivo*, this model will be an invaluable tool to evaluate the efficacy of *ex vivo* therapies, such as hematopoietic gene therapy, to restore the repopulating properties of HSCs defective in *DKC1*. Moreover, the repopulation defects observed in our study in DC-like HSPCs would suggest that the restored function of dyskerin through gene therapy strategies might confer a proliferation advantage in DC HSPCs, as we have already demonstrated in FA patients treated by hematopoietic gene therapy (27).

Aiming at restoring the function of X-DC cells, discrepant results have been observed after the ectopic expression of dyskerin (22, 28, 29). The use of codon optimized sequences of *DKC1* (not recognized by *DKC1*-shRNAs) or the use of functionally active *DKC1*-derived sequences, such as those encoding for GSE24.2 and GSE4 peptides (29-31), might compensate the molecular and cellular defects of DC HSCs. As proposed for FA (27), the correction of HSCs in early stages of the disease of DC would be also relevant to complement the function of affected genes before telomeres are significantly reduced.

Whether or not gene complementation in DC HSPCs with shortened telomeres would facilitate their elongation is currently unknown and will require extensive studies in this and other DC models.

Conclusion

The generation of DC-like HSPCs constitutes a new platform for studying the molecular basis of the BMF in DC and also for screening the efficacy and safety of hematopoietic therapies for DC patients, including gene therapy and drugs capable of protecting or restoring the function of DC HSPCs.

List Of Abbreviations

AlloHSCT: Allogeneic hematopoietic stem cell transplantation

BM: Bone marrow

BMF: Bone marrow failure

DC: Dyskeratosis congenita

FA: Fanconi anemia

HSC: Hematopoietic stem cell

HSPC: Hematopoietic stem and progenitor cell

LV: Lentiviral vector

shRNA: Short hairpin RNA

TBD: Telomere biology disorder

TERC: Telomerase RNA component

TERT: Telomerase reverse transcriptase

TRAP: Telomeric repeat amplification protocol

VCN: Vector copy number

WB: Western blot

X-DC: X-linked dyskeratosis congenita

Declarations

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Author Contribution: CCR: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; HAZ: conception and design, collection and/or assembly of data, data analysis and interpretation; LPB: collection and/or assembly of data; BFV: collection and/or assembly of data; MLL: collection and/or assembly of data; CMG: collection and/or assembly of data; LS: conception and design, data analysis and interpretation, manuscript writing; JAB: conception and design, data analysis and interpretation, financial support, manuscript writing; RP: conception and design, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript; GG: conception and design, collection and/or assembly of data, data analysis and interpretation, financial support, manuscript writing, final approval of manuscript.

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Availability of the data and materials: The authors confirm that the data supporting the findings of this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate: Human cord blood samples from healthy donors were kindly provided by the *Centro de Transfusión de la Comunidad de Madrid* under the approval of its IRB and in accordance with the Helsinki Declaration. In all instances, informed consents were previously signed by the donors. All experimental procedures involving mice were conducted at the CIEMAT animal facility (registration number 28079-21 A) and were approved by the Animal Welfare Body of this institution. This project was authorized by the competent authorities of the *Comunidad de Madrid*, under the registration number PROEX-70/15 fulfilling Spanish and European legislation (Spanish RD 53/2013 and Law 6/2013 in compliance with the European Directive 2010/63/EU about the use and protection of vertebrate mammals used for experimentation and other scientific purposes).

Consent for publication: Not applicable.

Competing interests: The authors declare that they have no competing, neither financial nor non-financial, interests.

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Figures

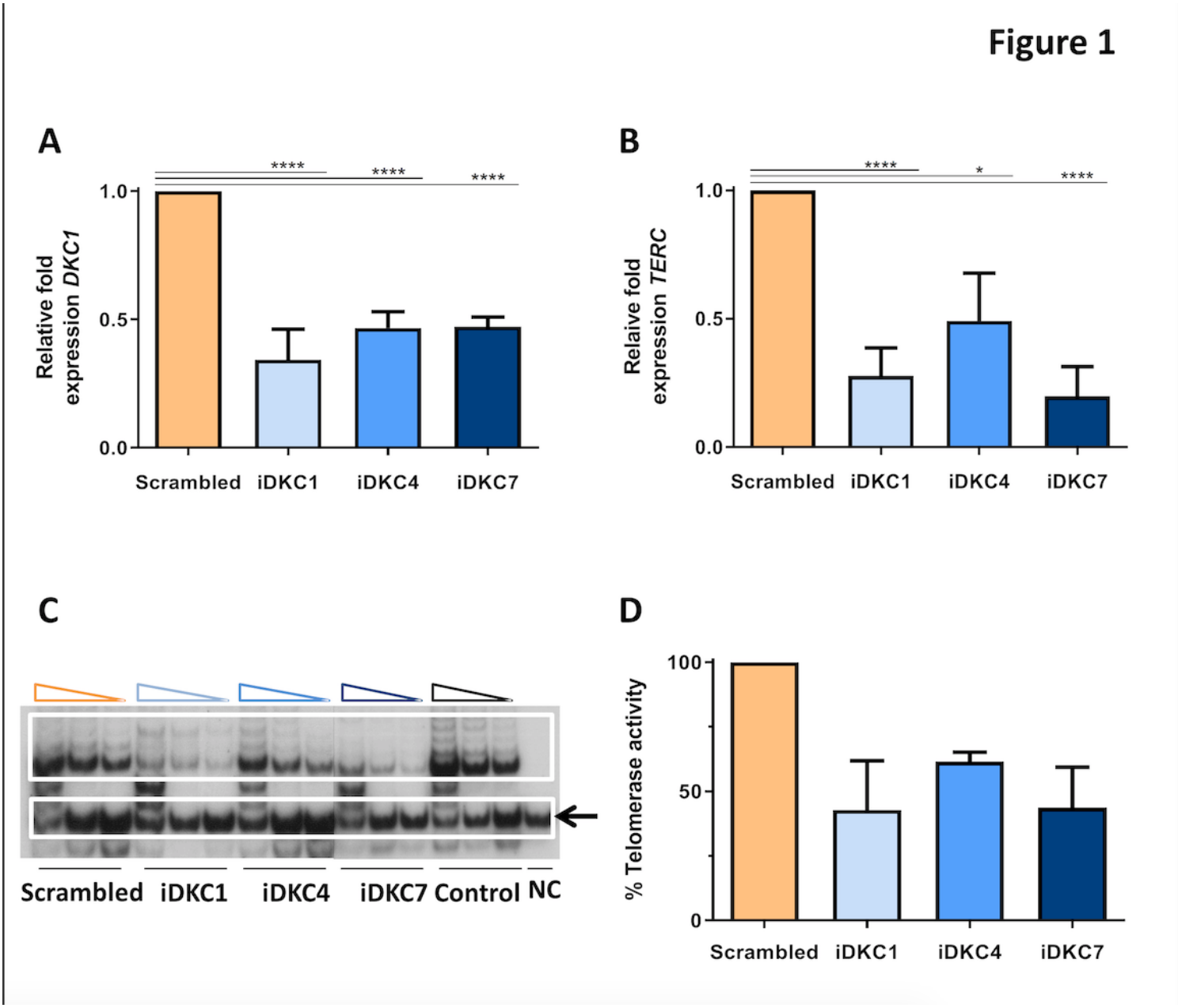

Figure 1

Molecular implications associated with the inhibition of DKC1 in human hematopoietic stem and progenitor cells. Cord blood CD34+ cells were transduced with specific anti shRNA-LVs and maintained in liquid cultures for 5-8 days (See details in materials and methods). A) Decreased expression of DKC1 gene after CD34+ cell transduction with specific shRNA-LVs (7 independent experiments were conducted; n=7). DKC1 expression levels in cells transduced with DKC1 shRNA LVs represent relative values of those obtained in cells transduced with the scrambled shRNA LV. B) Decreased expression of TERC after CD34+ cell transduction with specific shRNA-LVs (n=7). C) Representative analysis of a telomeric repeat amplification protocol (TRAP). Internal control is marked by the black arrow, negative control was performed with buffer (NC) and non-transduced cells were used as control. D) Analysis of the telomerase activity after transduction with shRNA LVs (n=3). Data are expressed as mean ± SEM. Asterisks indicate significant differences determined by Student's t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

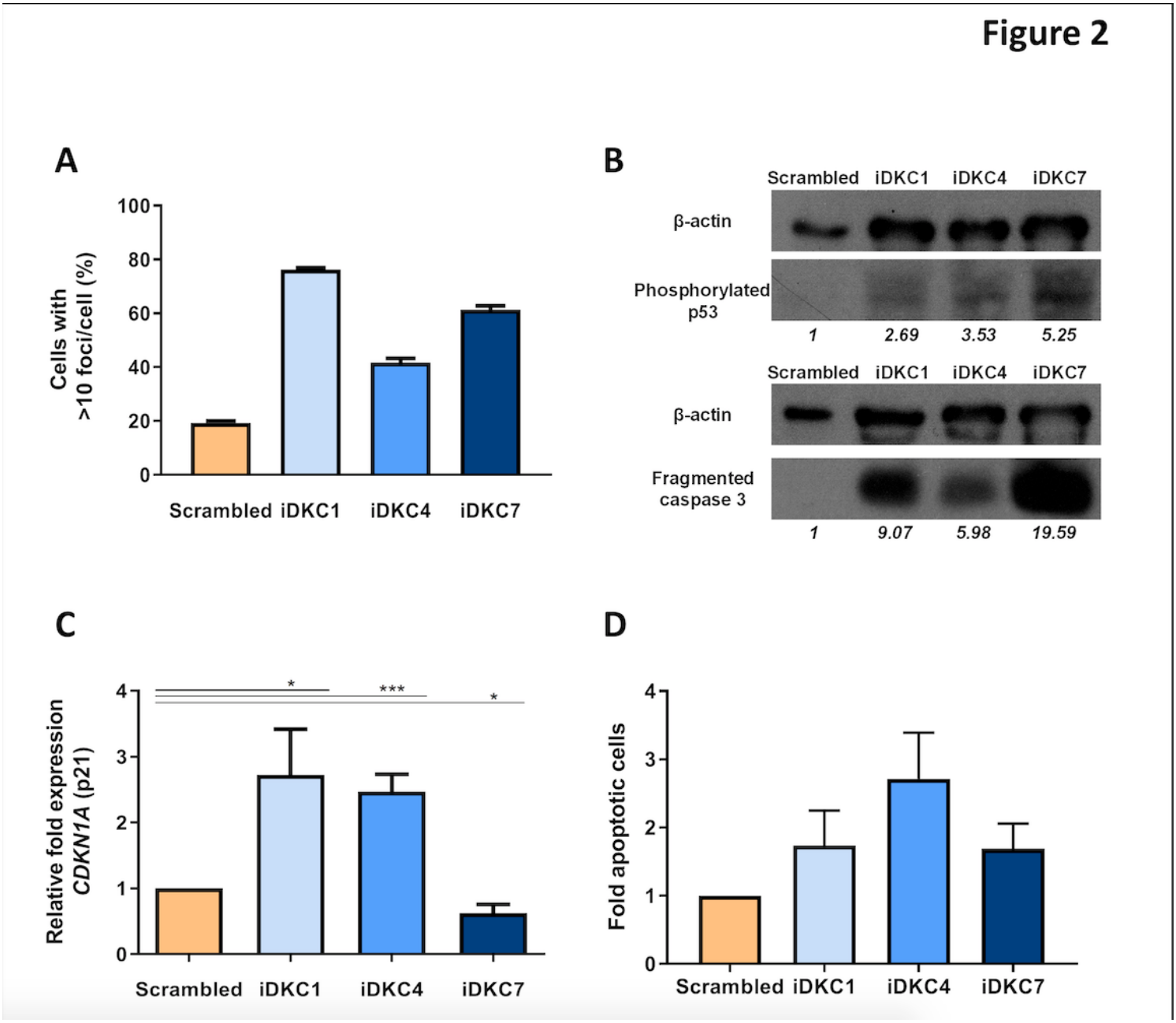

Figure 2

DNA damage and apoptosis associated with the inhibition of DKC1 in human hematopoietic stem and progenitor cells. Cord blood CD34+ cells were transduced with specific anti shRNA-LVs and maintained in liquid cultures for 10 days (See details in materials and methods). A) Analysis of DNA damage in CD34+ cells transduced with specific shRNA LVs. Cells with more than 10 γH2AX foci per cell are shown (n=3). B) Representative Western blot (WB) assays for phosphorylated p53 (upper WB) and fragmented caspase 3 (lower WB) expression using β-actin as control. Quantification appears in italics below the images as the ratio of expression in relation with the control protein. C) Increased expression of p21 after transduction of CD34+ cells with specific shRNA-LVs (n=6). D) Fold increase of apoptotic cells (Annexin V+) in comparison with the scrambled control condition (n=3). Data are expressed as mean ± SEM.

Asterisks indicate significant differences determined by Student's t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

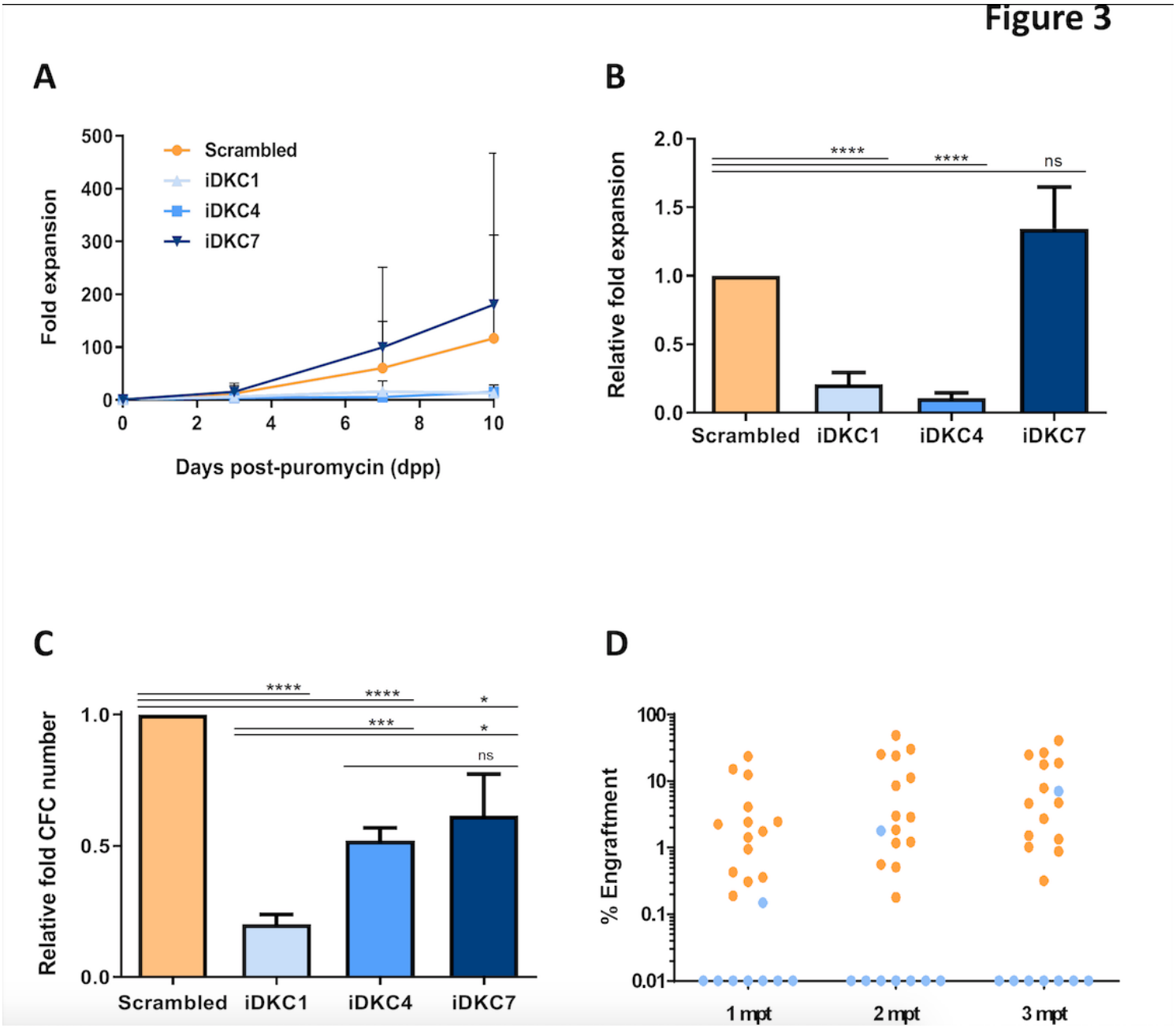

Figure 3

Analysis of the in vitro growth properties and in vivo repopulating ability of DKC1-interfered CD34+ cells. A) Analysis of the cell expansion analyzed 2 weeks after ex vivo incubation of transduced cells in liquid culture (n=5). B) Relative cell expansion in comparison with cells transduced with the scrambled shRNA-LV (n=6). C) Analysis of the clonogenic potential of CD34+ cells transduced with DKC1-shRNA LVs and scrambled shRNA-LVs (n = 8). D) Analysis of the repopulation potential of CD34+ cells transduced with scrambled shRNA-LV (orange dots) or DKC1-shRNA LVs (blue dots). The proportion of human CD45+ cells in the BM of recipient mice was analyzed at 1-3 months post-transplantation (mpt). Data are

expressed as mean ± SEM. Asterisks indicate significant differences determined by Student's t test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

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