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

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Abstract

Dyskeratosis congenita 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 dyskeratosis congenita patients is allogeneic hematopoietic stem cell transplantation. However due to the toxicity associated to allogeneic hematopoietic stem cell transplantation in dyskeratosis congenita, new nontoxic therapies are recommended to improve the life expectancy of these patients. Since bone marrow biopsies are not routinely performed during the follow-up of dyskeratosis congenita patients, the availability of dyskeratosis congenita hematopoietic stem cells constitutes a major limitation in the development of new hematopoietic therapies for dyskeratosis congenita. Here we aimed at generating dyskeratosis congenita-like hematopoietic stem cells in which the efficacy of new therapies could be investigated. X-linked dyskeratosis congenita is one of the most frequent variants of dyskeratosis congenita and is associated with an impaired expression of the DKC1 gene. In this study we thus generated dyskeratosis congenita-like hematopoietic stem cells based on the stable knock-down of DKC1 in human CD34+ cells, using 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. Moreover, DKC1-interfered human CD34+ cells showed defective clonogenic ability and were incapable of repopulating the hematopoiesis of immunodeficient NSG mice. The development of dyskeratosis congenita-like hematopoietic stem cells will facilitate the understanding of the molecular and cellular basis of the bone marrow failure characteristic of dyskeratosis congenita patients, and will serve as a platform for the development of new hematopoietic therapies for dyskeratosis congenita patients.

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, TCA81, 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 FA (18), would be highly beneficial for DC patients.
Materials And Methods

Human hematopoietic cells

Mononuclear cells from pooled umbilical cord blood samples (CB) were obtained through a density gradient using Ficoll-Paque (GE Healthcare) and CD34+ cells were purified using the MACS CD34 Micro-Bead kit (Miltenyi Biotec). Umbilical CB samples were kindly provided by the Centro de Transfusión de la Comunidad de Madrid. In all instances informed consents were previously signed from the mothers.

Cells were cultured in StemSpam (StemCell Technologies) medium supplemented with 1% GlutaMAX (Gibco), 1% penicillin/streptomycin solution (Gibco), 100 ng/ml hTPO, hFIT3L and hSCF and 20 ng/ml IL-3 (all growth factors from EuroBiosciences).

Lentiviral vectors and generation of human X-DC-like HSPCS

Second-generation self-inactivating lentiviral vectors (LVs) were generated in HEK 293T cells. These cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich) and 0.5% penicillin/streptomycin (P/S, Gibco). Supernatants were collected at 24 and 48 hours post-transfection and viruses were concentrated by centrifugation at 50000g for 2 hours. Titters were determined by flow cytometry or quantitative polymerase chain reaction (qPCR) in 293T cells, plated at 5x10^4 cells per well in 24-well plates and infected with serial dilutions of LVs overnight, after 14 days of culture.

LVs titration was performed in HEK 293T cultured in DMEM (Gibco) supplemented as previously described. Cells were transduced with serial dilutions of the corresponding LV. When the LV carried the EGFP marker, cells were analyzed by flow cytometry after 5-12 days of liquid culture. In the remaining cases, titration was conducted by qPCR in an Applied 7500 Fast Real Time PCR system (Thermo Fisher Scientific).

Seven short hairpin RNAs (shRNAs) were respectively inserted into the pLKO.1 LV (GE Healthcare Life Sciences), which also carry the puromycin resistance gene (Suppl. Table 1). A pGIPZ LV with a scrambled shRNA sequence was used as control. To generate DC-like HSPCS, healthy CD34+ cells were transduced with these LVs at a MOI of 100 IU/cell as previously described (1). Two cycles of transduction were performed to increase the percentage of transduction and the vector copy number (VCN). In all instances, transduced CD34+ cells with LVs-encoding the different shRNAs were selected for 72h in liquid cultures containing 1 µg/ml of puromycin to prevent the generation of untransduced CD34+ cells. Under these conditions no hematopoietic colonies were generated by untransduced samples.

Analysis of lentiviral vector copy number

When CD34+ cells were analyzed, samples were maintained in vitro for 2 weeks to prevent the analysis of non-integrated copies of the LV. DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen). Genomic DNA from hematopoietic colonies was obtained by adding 20 mL of lysis buffer (0.3 mM Tris HCl, pH 7.5; 0.6 mM CaCl2; 1.5% Glycerol; 0.675% Tween-20; and 0.3 mg/mL Proteinase K). Lysis protocol consists on 30 minutes at 65°C, 10 minutes at 90°C and 10 minutes at 4°C. LV vector copy number (VCN) was quantified by qPCR detection of Psi sequence (specific primers for Psi were Psi forward (Fw) 5’-CAGGACTCGGCTTGGCTGAAG-3’ and Psi reverse (Rv) 5’-TCCCTTATCTGACGTAC-3’ with a Taqman probe Psi FAM 5’-CGCACGGCAAGGCGGAG-3’) normalized to endogenous human albumin gene (hAlb) (specific primers for hAlb were Alb Fw 5’-
GCTGTCATCTTTTGGGCTG-3’ and Alb Rv 5’-ACTCATGGGAGCTGCTGGTTC-3’. qPCR was performed in an Applied 7500 Fast Real Time PCR system (Thermo Fisher Scientific), as previously described (2).

**Gene expression assays**

DKC1-interfered CD34+ cells were pelleted and RNA was extracted using TRIzol Reagent (Invitrogen). The expression of DKC1, TERC and CDKN1A mRNA was analyzed by real-time quantitative reverse transcriptase-PCR (qRT-PCR) of cDNA obtained from total RNA from DKC1-interfered CD34+ cells. Untransduced samples and samples transduced with scrambled LV were used as negative controls.

The relative expression of DKC1, TERC and p21 (CDKN1A) was determined in a StepOne Plus Real-Time PCR System (Applied Biosystems) by the 2^-ΔΔCt method as previously described (3), using Power SYBR Green kit (Applied Biosystems) and the following primers: DKC1 forward (Fw) 5’-GCTCCCTCAGTTGATCAAGAAGG-3’ and reverse (Rv) 5’-CTCAGAAAACCAATTCTACCTC-3’, TERC Fw 5’-TCTAACCCTAAGAAGGCCG TGAG-3’ and Rv 5’-GTTTGCCTCTAGATGACGCTG-3’ and CDKN1A (p21) Fw 5’-GCTGCAGGGGACAGCA GAG-3’ and Rv 5’-GCTTCCCTTGGGAGAAAGATCAG-3’. For housekeeping control expression human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used Fw 5’-GAGAGACCCTCACTGCTG-3’ and Rv 5’-GATGGTACATGACAAGGTGG-3’.

**Telomeric repeat amplification protocol assay**

Telomerase activity was determined under recommended standard conditions of the TRAPEZE Telomerase Detection S7700 Kit (Millipore) for telomeric repeat amplification protocol (TRAP) using radioisotopic detection. Telomerase activity was determined in each sample using 0.5, 0.25 and 0.125 μg of protein extract and normalized with the internal control included in the assay (4, 5).

**Analysis of DNA damage**

CD34+ cells were maintained 24 hours in 4-chambers Falcon CultureSlides (Corning) pre-coated with 5 μg/cm2 of Retronectin (Takara). DNA damage was analyzed by determining and quantifying histone γH2AX foci. The γH2AX localization was carried out by fluorescence microscopy. For this purpose, cells were fixed in 3.7% formaldehyde solution (Fluka, Sigma) at room temperature for 15 min. After washing with 1x PBS, cells were permeabilized with 0.2% Triton X-100 in PBS and blocked with 10% horse serum before overnight incubation with γH2AX antibody. Finally, cells were washed and incubated with secondary antibodies coupled to fluorescent dyes (Alexa Fluor 488 or/and Alexa Fluor 647). Images were acquired with a Confocal Spectral Leica TCS SP5 using a HCX PL APO lambda blue 63x/1.4 oil UV, zoom 2.3 lens. Images were acquired using LAS-AF 1.8.1 Leica software and processed using LAS-AF 1.8.1 Leica software and Adobe Photoshop CS. At least 200 cells were evaluated for γH2A.X staining.

**Colony-Forming Cell Assays**

To determine the number of colony-forming cells (CFCs), DKC1-interfered CD34+ cells were cultured in 35 mm plates in methylcellulose medium (Methocult) for 14 days at 37°C, 5% CO₂ and 90% relative humidity. After that time, granulocyte-macrophage colony-forming units (GM-CFU) and erythroid burst-forming units (E-BFU) were quantified using an inverted microscope (Nikon).

**Telomere length**

The relative telomere length was assessed by quantitative Polymerase Chain Reaction (qPCR) as previously described (6). The qPCR determines the ratio of telomere (T) repeat copy number to single-copy (S) gene (36B4) copy number (T/S ratio) in experimental samples, as compared with a reference DNA sample (MCF7 cells).

**Hematopoietic Reconstitution potential**
NOD-SCID-Il2rg<sup>-/-</sup> (NSG) mice were obtained from the Jackson Laboratory and maintained at the CIEMAT animal facilities (registration 28079-21 A). All the experimental procedures fulfill the Directive 2010/63/UE about the use and protection of mammals used for experimentation and other scientific purposes. NSG mice were previously irradiated with 1.5 Gy (X-rays; MG324 Philips) 24 hours before intravenous HSC transplantation through retro-orbital sinus. Human hematopoietic engraftments were evaluated by performing femoral bone marrow (BM) aspirations at 1 and 2 months post-transplant (mpt). After 3 mpt, transplanted mice were culled and femoral BM cells were obtained. Samples were analyzed by flow cytometry using hCD45 (304014, BioLegend), hCD33 (A07775, Beckman Coulter), hCD19 (25-0198-42, eBioscience) and hCD34 (555824, Becton Dickinson) monoclonal antibodies according to the manufacturer’s instructions. All these analysis were performed on the LSR Fortessa (BD Biosciences) and processed with FlowJo V10 software (FlowJo, LLC).

**Statistics**

All statistical analyses were performed using GraphPad Prism 7.00. Results are reported as mean ± standard error of mean (SEM) and significance was set as p ≤ 0.05 throughout the experiments. Statistical differences between means were evaluated by Student’s t test after checking the Gaussian distribution of the sampled data using Shapiro-Wilk normality test.

**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-regulated expression 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).

Seven different shRNA LVs were first screened for in healthy donor CD34<sup>+</sup> cells. In subsequent experiments we demonstrated that three of these shRNA-LVs, iDKC1, iDKC4 and iDKC7, significantly decreased DKC1 mRNA levels to 40-47% compared to levels determined in cells transduced with the scrambled shRNA LV (Figure 1A and Suppl. Figure 1A). 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 of inhibiting DKC1, we evaluated the expression of TERC in CD34<sup>+</sup> cells transduced with scrambled and DKC1-shRNA LVs. As shown in Figure 1B, TERC mRNA levels in cells transduced with iDKC1, iDKC4 or iDKC7 LVs were respectively decreased to 27%±11%, 49%±19% and 19%±0.11% compared to levels determined in the control group. In subsequent analyses, changes in the telomerase functionality of DKC1-interfered CD34<sup>+</sup> cells were quantified. To this end, we measured telomerase activity of DKC1-interfered and control CD34<sup>+</sup> cells by TRAP assay. These results showed significant decreases in the telomerase activity of DKC1-interfered cells to 42.8%±33.0, 61.5%±6.3% and 43.7%±27.19% in CD34<sup>+</sup> cells transduced with iDKC1, iDKC4 or iDKC7 LVs, respectively (Figures 1C and Figure 1D).

In the following experiments we investigated the implication of DKC1 interference in the DNA damage of CD34<sup>+</sup> cells. Analyses of γH2AX foci in the nucleus of cells transduced with iDKC1, iDKC4 or iDKC7 LVs revealed that, while only 19% of cells transduced with the scrambled shRNA LV showed more than 10 γH2AX foci per cell, a significantly increased proportion of CD34<sup>+</sup> cells with γH2AX foci was observed in cells transduced with either the iDKC1 (76%), iDKC4 (42%) or the iDKC7 (61%) LVs (Figure 1E). In next studies we determined the expression of p21 (CDKN1A) in CD34<sup>+</sup> cells transduced with the different constructs. As shown in Figure 1F, iDKC1 and iDKC4 LVs, but not iDKC7, significantly increased the expression of p21 (2.7±1.6 and 2.3±0.53 fold, respectively, compared to the control group), suggesting the induction of cell cycle arrest and probably
apoptosis in DKC1-interfered HSPCs.

Taken together, these molecular studies showed that the down-regulated expression of DKC1 inhibits the telomerase activity and increases DNA damage and cell senescence in human HSPCs.

**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 expanded for 10 days (see Materials and Methods). While cells transduced with the scrambled shRNA LV showed a marked expansion during this period (117±87.31 fold compared to initial cell numbers), the expansion observed in iDKC1- and iDKC4-transduced CD34+ cells was significantly inhibited. In these cases, expansions were limited to 13±6.99 and 15.3±2.42 fold compared to initial numbers of CD34+ cells (Figure 2A). These values represented 25±19% and 12±10%, respectively, the expansion observed in the control group (CD34+ cells transduced with the scrambled shRNA LV) (Figure 2B). Telomere length in DKC1-interfered cells was measured and no evident changes were observed, probably due to the limited number of cell cycles which took place in these cultures (See Suppl. Figure 2).

**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 the telomerase activity. As also observed in cells from DC patients, DKC1 inhibition was linked to markers of DNA damage, such as the generation of nuclear γH2AX foci and up-regulation of p21 expression.

Hematopoietic studies in DC patients showed reduced numbers of HSPCs in their BM (26). Consistent with these observations, DKC1 inhibition markedly reduced the expansion, as well as the clonogenic potential of CD34+ cells. Remarkably, these effects were evident immediately after DKC1 interference, despite of detecting no changes in the telomere length of these cells. This observation indicates that the inhibited proliferation of DC-like HSPCs, and very probably from X-DC patients, is not necessarily a consequence of the reduced telomere length, suggesting that cell defects such as DNA damage and apoptosis would account for the proliferating defects of DC HSPCs.

Remarkably, our study also demonstrates that the down-regulated expression of DKC1 is sufficient to ablate the in vivo repopulating properties of human HSCs, even before the generation of changes in the telomere length of these cells. The repopulation defects observed in DC-like HSPCs strongly suggests that the restored function of dyskerin might confer a proliferation advantage in DC HSPCs, as demonstrated in FA patients treated by 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 will 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.

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
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, final approval of manuscript; 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 carried out according to Spanish and European legislation (Spanish RD 53/2013 and Law 6/2013 in compliance with the European Directive 2010/63/UE about the use and protection of vertebrate mammals used for experimentation and other scientific purposes).

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### References

1. Meyne J, Ratliff RL, Moyzis RK. Conservation of the human telomere sequence (TTAGGG)n among vertebrates. Proc Natl Acad Sci U S A. 1989 Sep;86(18):7049-53. PubMed PMID: 2780561. Pubmed Central PMCID: PMC297991.
2. Savage SA. Human telomeres and telomere biology disorders. Prog Mol Biol Transl Sci. 2014;125:41-66. PubMed PMID: 24993697.
3. Jones M, Bisht K, Savage SA, Nandakumar J, Keegan CE, Maillard I. The shelterin complex and hematopoiesis. J Clin Invest. 2016 May 2;126(5):1621-9. PubMed PMID: 27135879. Pubmed Central PMCID: PMC4855927.
4. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell. 1985 Dec;43(2 Pt 1):405-13. PubMed PMID: 3907856.
5. Blackburn EH. Telomeres and telomerase: their mechanisms of action and the effects of altering their functions. FEBS Lett. 2005 Feb 7;579(4):859-62. PubMed PMID: 15680963.
6. Cohen SB, Graham ME, Lovrecz GO, Bache N, Robinson PJ, Reddel RR. Protein composition of catalytically active human telomerase from immortal cells. Science. 2007 Mar 30;315(5820):1850-3. PubMed PMID: 17395830.
7. Kirwan M, Dokal I. Dyskeratosis congenita: a genetic disorder of many faces. Clin Genet. 2008 Feb;73(2):103-12. PubMed PMID: 18005359.
8. Townsley DM, Dumitriu B, Young NS. Bone marrow failure and the telomeropathies. Blood. 2014 Oct 30;124(18):2775-83. PubMed PMID: 25237198. Pubmed Central PMCID: PMC4215309.
9. Dokal I, Valliamy T, Mason P, Bessler M. Clinical utility gene card for: Dyskeratosis congenita - update 2015. Eur J Hum Genet. 2015 Apr;23(4). PubMed PMID: 25182133. Pubmed Central PMCID: PMC4667501.
10. Perdigones N, Perin JC, Schiano I, Nicholas P, Biegel JA, Mason PJ, et al. Clonal hematopoiesis in patients with dyskeratosis congenita. Am J Hematol. 2016 Dec;91(12):1227-33. PubMed PMID: 27622320. Pubmed Central PMCID: PMC5118079.
11. Perona R, Iarriccio L, Pintado-Berninches L, Rodriguez-Centeno J, Manguan-Garcia C, Garcia E, et al. Molecular Diagnosis and Precision Therapeutic Approaches for Telomere Biology Disorders. In: Larramendy ML, editor. Telomere - A Complex End of a Chromosome. Rijeka: InTech; 2016. p. Ch. 05.
12. Savage SA, Dufour C. Classical inherited bone marrow failure syndromes with high risk for
myelodysplastic syndrome and acute myelogenous leukemia. Semin Hematol. 2017 Apr;54(2):105-14. PubMed PMID: 28637614.

13. Dokal I, Vulliamy T. Inherited bone marrow failure syndromes. Haematologica. 2010 Aug;95(8):1236-40. PubMed PMID: 20675743. Pubmed Central PMCID: PMC2913069.

14. He J, Navarrete S, Jasinski M, Vulliamy T, Dokal I, Bessler M, et al. Targeted disruption of Dkc1, the gene mutated in X-linked dyskeratosis congenita, causes embryonic lethality in mice. Oncogene. 2002 Oct 31;21(50):7740-4. PubMed PMID: 12400016.

15. Calado RT, Regal JA, Hills M, Yewdell WT, Dalmazzo LF, Zago MA, et al. Constitutional hypomorphic telomerase mutations in patients with acute myeloid leukemia. Proc Natl Acad Sci U S A. 2009 Jan 27;106(4):1187-92. PubMed PMID: 19147845. Pubmed Central PMCID: PMC2627806.

16. Fernandez Garcia MS, Teruya-Feldstein J. The diagnosis and treatment of dyskeratosis congenita: a review. J Blood Med. 2014;5:157-67. PubMed PMID: 25170286. Pubmed Central PMCID: PMC4145822.

17. Barbaro P, Vedi A. Survival after Hematopoietic Stem Cell Transplant in Patients with Dyskeratosis Congenita: Systematic Review of the Literature. Biol Blood Marrow Transplant. 2016 Jul;22(7):1152-8. PubMed PMID: 26968789.

18. Rio P, Navarro S, Wang W, Sanchez-Dominguez R, Pujol RM, Segovia JC, et al. Successful engraftment of gene-corrected hematopoietic stem cells in non-conditioned patients with Fanconi anemia. Nat Med. 2019 Sep;25(9):1396-401. PubMed PMID: 31501599.

19. Autexier C. POT of gold: modeling dyskeratosis congenita in the mouse. Genes Dev. 2008 Jul 1;22(13):1731-6. PubMed PMID: 18593874. Pubmed Central PMCID: PMC2732423.

20. Kirwan M, Dokal I. Dyskeratosis congenita, stem cells and telomeres. Biochimica et biophysica acta. 2009;1792(4):371-9. PubMed PMID: 19419704.

21. Hockemeyer D, Palm W, Wang RC, Couto SS, de Lange T. Engineered telomere degradation models dyskeratosis congenita. Genes & Development. 2008;22(13):1773-85.

22. Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. Nature. 1999 12/02/online;402:551.

23. Parry EM, Alder JK, Lee SS, Phillips JA, 3rd, Loyd JE, Duggal P, et al. Decreased dyskerin levels as a mechanism of telomere shortening in X-linked dyskeratosis congenita. J Med Genet. 2011 May;48(5):327-33. PubMed PMID: 21415081. Pubmed Central PMCID: PMC3088476.

24. Cong YS, Wright WE, Shay JW. Human Telomerase and Its Regulation. Microbiology and Molecular Biology Reviews. 2002;66(3):407-25.

25. Marrone A, Stevens D, Vulliamy T, Dokal I, Mason PJ. Heterozygous telomerase RNA mutations found in dyskeratosis congenita and aplastic anemia reduce telomerase activity via haploinsufficiency. Blood. 2004;104(13):3936-42.

26. Frederick D, Goldman GA, Al J, Klingelhutz, Mark Hills, Sarah R. Cooper, Wendy S. Hamilton, Annette J. Schlueter, Karen Lambie, Connie J. Eaves and Peter M. Lansdorp. Characteristics of primitive hematopoietic cells from patients with Dyskeratosis congenita. Hematopoiesis and Stem Cells. 2008;111(9):4523 - 31.

27. Rio P, Navarro S, Guenechea G, Sanchez-Dominguez R, Lamana ML, Yanez R, et al. Engraftment and in vivo proliferation advantage of gene-corrected mobilized CD34(+) cells from Fanconi anemia patients. Blood. 2017 Sep 28;130(13):1535-42. PubMed PMID: 28801449.

28. Bellodi C, McMahon M, Contreras A, Juliano D, Kopmar N, Nakamura T, et al. H/ACA Small RNA Dysfunctions in Disease Reveal Key Roles for Noncoding RNA Modifications in Hematopoietic Stem Cell Differentiation. Cell Reports. 2013;3(5):1493-502.

29. Machado-Pinilla R, Sanchez-Perez I, Murguia JR, Sastre L, Perona R. A dyskerin motif reactivates telomerase activity in X-linked dyskeratosis congenita and in telomerase-deficient human cells. Blood. 2008 Mar 1;111(5):2606-14. PubMed PMID: 18057229.

30. Iarriccio L, Manguan-Garcia C, Pintado-Berninches L, Mancheno JM, Molina A, Perona R, et al. GSE4, a Small Dyskerin- and GSE24.2-Related Peptide, Induces Telomerase Activity, Cell Proliferation and Reduces DNA Damage, Oxidative Stress and Cell Senescence in Dyskerin Mutant Cells. PloS one. 2015;10(11):e0142980. PubMed PMID: 26571381. Pubmed Central PMCID: PMC4646510.

31. Manguan-Garcia C, Pintado-Berninches L, Carrillo J, Machado-Pinilla R, Sastre L, Perez-Quilis C, et al. Expression of the genetic suppressor element 24.2 (GSE24.2) decreases DNA damage and oxidative stress in X-linked dyskeratosis congenita cells. PloS one. 2014;9(7):e101424. PubMed PMID: 24987982.
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). E) 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). F) Increased expression of p21 after transduction of CD34+ cells with specific shRNA-LVs (n=6). 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

Analysis of the in vitro growth properties and in vivo repopulating ability of DKC1-interfered CD34+ cells. A) Analysis of the cell expansion analyzed 13 days 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).

Supplementary Files

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