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Cord Blood

Influence of Donor and Recipient Gender on Telomere Maintenance after Umbilical Cord Blood Cell Transplantation: A Study by the Gruppo Italiano Trapianto Di Midollo Osseo

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A B S T R A C T

Physiologic loss of telomerase activity in adult life determines progressive telomere length (TL) shortening. Inflammation and oxidative damage are established causes of TL loss; moreover, males have shorter telomeres compared with females. Despite these notions, mechanisms regulating TL maintenance are poorly defined. Because umbilical cord blood (UCB) cells harbor very long telomeres, not yet exposed to environmental damages, UCB transplantation (UCBT) provides a unique experimental setting to study determinants of TL in humans. TL dynamics were analyzed on peripheral blood mononuclear cells (MNCs) from 36 patients (median age, 42 years) undergoing UCBT. TL was studied at a median of 20 months after UCBT. A significantly longer TL (mean, 8698 bp; range, 6521 to 11,960) was documented in UCBT recipients compared with age-matched healthy control subjects (mean, 7396 bp; range, 4375 to 11,108; $P < .01$). Among variables potentially influencing TL maintenance, including recipient features, graft type, transplant procedure, and engraftment kinetics, only donor–recipient gender combination was associated with TL, with the longest TL in women receiving male UCB (mean, 10,063 bp; range, 8381 to 11,960). To further investigate this trend, telomerase activation was assessed in vitro. Experiments showed that telomerase subunits were preferentially upregulated in male-derived bone marrow MNCs exposed ex vivo to estradiol as compared with female MNCs. This implies an increased sensitivity of male-derived MNCs to telomerase activation induced by estradiol. The results suggest that extrinsic and modifiable factors such as hormonal status and female milieu could be major determinants of TL in humans, providing the rationale for investigating hormonal-based approaches to counteract telomere erosion and aging-related diseases.

INTRODUCTION

Telomeres are noncoding long-tandem-repeated sequences at the ends of chromosomes that prevent chromosomal fusions, accumulation of DNA damage, and degradation of coding DNA [1,2]. Telomere maintenance is crucial for genomic stability in eukaryotic cells, because telomere shortening leads to accumulation of DNA damage and replicative senescence or cell death [2]. Telomere shortening is prevented by human

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telomerase (hTERT), an enzyme able to add nucleotides pairs de novo, restoring telomere sequences [3–7]. hTERT downregulation is a common event in adults, leading to progressive telomere shortening over time on each round of cell division [2–6,8]. Human leukocyte telomere length (TL) correlates with lifespan and has been extensively studied as a biomarker for cancer and age-related diseases, such as diabetes, atherosclerosis, and cancer [2,8–10].

Established causes of telomere attrition include reactive oxygen species–mediated DNA damage and systemic inflammation (probably through reactive oxygen species generation) [1,11]. In fact, telomeres are very sensitive to DNA damage, because the efficiency of DNA repair mechanisms in telomeres is suboptimal [12,13]. Furthermore, in adults telomeres are shorter in men compared with women, and, accordingly, men have shorter life expectancy and higher cancer incidence [1,14,15].

Several studies indicate that high proliferative stresses, including bone marrow (BM) regeneration after hematopoietic stem cell transplantation, produce a nonphysiologic TL shortening. In fact, as a consequence of an increased number of replicative cycles during engraftment, leukocyte TL is significantly decreased in both autologous and allogeneic hematopoietic stem cell transplantation compared with either pretransplant or donor TL measurements, respectively [16–18]. Besides graft-versus-host disease (GVHD), which has been correlated with TL as a source of systemic inflammation and oxidative stress, determinants of TL in BM transplant (BMT) recipients are still poorly defined.

Umbilical cord blood transplant (UCBT) represents an ideal setting to investigate determinants of TL. In fact, in UCBT the stem cell source is characterized by very long telomeres not yet exposed to environmental factors. Additionally, there is a lower chronic GVHD incidence compared with hematopoietic stem cell transplantation with other allogeneic stem cell sources [19].

In the present study we evaluated TL in 36 adult subjects surviving long term after UCBT. We first confirmed previous findings observing an overall longer TL after UCBT compared with age-matched healthy control subjects [8,19]. Furthermore, we showed that among several clinical factors, only recipient and donor gender significantly correlated with TL, with female recipients retaining longer telomeres compared with male recipients. Notably, the longest telomeres were observed in women transplanted with male UCB, suggesting that male-derived stem cells may retain long telomeres if placed in a female milieu. In line with this hypothesis, ex vivo treatment with estradiol (E_2) resulted in a preferential upregulation of telomerase subunits (*TERT*, *TERC*, *DKC1*) in male-derived BM mononuclear cells (MNCs) compared with their female counterparts. Taken together, these data indicate that UCBT provides a stem cell source able to sustain and rejuvenate human hematopoiesis, extrinsic and modifiable factors such as hormonal status and female milieu are major determinants of TL in the transplant setting, and E_2-mediated telomerase upregulation may prevent telomere erosion in men. These observations could have broad implications for our understanding of telomere dynamics, providing the rationale for further clinical investigation of hormonal therapy to prevent telomere erosion–related diseases and aging.

METHODS

Patients

The study was performed on peripheral blood (PB) cells from 36 subjects who received UCBT between 2008 and 2013 at 12 Italian Institutions belonging to the Gruppo Italiano Trapianto di Midollo Osseo. A standard transplant procedure was used in 23 subjects, whereas 13 had intrabone UCBT [20]. TL was assessed at a median of 19.5 months after UCBT to minimize differences arising from the known telomere modifications occurring during the first year after hematopoietic stem transplantation [10–12]. UCBT subjects were selected for complete chimerism and for stable hematologic reconstitution after UCBT. In addition, all patients were in remission of their hematologic disease and had not received any additional chemoradiotherapy after transplantation. The main characteristics of 36 UCBT subjects are reported in Table 1. As control, TL was determined on PB cells from 85 healthy volunteers, matched for age with UCBT subjects. Finally, TL was evaluated on cells from 46 UCB samples.

All patients and healthy volunteers gave a written informed consent for PB collection. This retrospective, noninterventional study on MNC-TL in subjects undergoing TL-modifying procedures was approved by our institutional review board (approval no. 602, May 12, 2013, Torino, Italy).

Part of the study included telomerase subunits gene expression assessment on normal BM cells after in vitro exposure to estrogenic stimulation. For this purpose, on written informed consent, primary BM MNCs were harvested from patients undergoing BM examination as part of diagnostic procedures. Samples with a negative result for any overt BM alteration were then used for the in vitro assay as described below. Also, this part of the study was also approved by our institutional review board (approval no. R633/17-IEO669, Milan, Italy).

Separation of MNCs

TL was assessed on PB or BM cells, after enrichment for MNCs, obtained through a density gradient separation (Ficoll-Paque; GE Healthcare, Buckinghamshire, UK).

Table 1
Main Patient Features and Transplant Procedure

Parameter	Value
Gender	
Female	22
Male	14
Age, yr	
Median (range)	42.5 (5-69)
Weight, kg	
Median (range)	58 (14-89)
Disease status at transplant	
CR/PR	21/8
NR	5
NA/NE	1/1
Procedure of transplantation	
Standard i.v./intrabone	23/13
Single UCB/double UCB	35/1
MNCs infused, $\times 10^7$/kg	
Median (range)	3.35 (.6-7.9)
$CD34^+$ cells infused, $\times 10^5$/kg	
Median (range)	1.46 (.10-5.6)
Time from UCBT to TL assessment, mo	
Median (range)	19.5 (1.6-139)
CBC at telomere assessment, median (range)	
WBCs, $\times 10^3$/mmc	6.7 (2.1-18.8)
Hb, gr%	12.4 (8.7-14.3)
PLTs, $\times 10^3$/mmc	194 (33-420)
Conditioning regimen	
Myeloablative	32/36
Reduced intensity	4/36
Disease	
Acute lymphoblastic leukemia	7
Acute myeloid leukemia/myelodysplasia	14/2
Chronic myeloproliferative neoplasms	3
Lymphoma/multiple myeloma	6/2
Thalassemia/Fanconi anemia	1/1
HLA match	
4/6	21
5/6	13
6/6	2

CR indicates complete response; PR, partial response; NR, nonresponder; NA, not applicable; CBC, complete blood count.

TL Assessment

TL was assessed by Southern blot analysis [21]. Briefly, 2 μg DNA were digested by mixing with *Hinf*I (20 U) and *Rsa*I (20 U) for 2 hours at 37°C, according to manufacturer's recommendations (Roche Diagnostic, Mannheim, Germany). Digested DNA fragments were then separated by .8% agarose gel electrophoresis in 1 TAE running buffer 1× (pH 8.0). After electrophoresis, gels were transferred to a positively charged nylon membrane (Roche Diagnostic) and then exposed for 10 seconds to UV light to fix DNA fragments. The TeloTAGGG Telomere Length Assay Kit (Roche Diagnostic) was used for the hybridization phase, in accordance with the manufacturer's instructions. Membranes were prehybridized for 2 hours in prehybridization solution at 62°C and then hybridized for 3 hours in the same conditions, adding 2 μL digoxigenin-labeled probe specific for telomeric repeats.

After hybridization, filters were washed twice at room temperature for 15 minutes in 2× washing solution and then twice at 39°C in .5× washing solution for 20 minutes. Filters were then incubated with a digoxigenin-specific antibody covalently coupled to alkaline phosphate. Finally, results were visualized using alkaline phosphate metabolizing CDP-Star, a highly sensitive chemiluminescent substrate. The light signal produced on the site of the hybridized probe was recorded on x-ray films (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostic). Chemiluminescence was detected by x-ray film (Roche Diagnostic) and scanned for analysis. Analysis was performed using Quantity One 4.6.3 (Bio-Rad Laboratories, Hercules, CA). Telomeres were visualized as smears, and the software calculated the intensity curve corresponding to each sample, and the point of maximum signal intensity defined the highest concentration of telomeric repeats ("peak telomere restriction fragment length"). Telomere restriction fragment length was determined by comparing the signals relative to standard molecular weights.

In Vitro Quantitative PCR Methods

To investigate the role of estrogens in stimulating telomerase activity and the possible difference in estrogen sensitivity of male versus female cells, BM MNCs from age-matched female and male volunteer donors were assayed in vitro. Briefly, on written informed consent primary BM MNCs from lymphoma patients undergoing initial staging procedures (who then resulted negative for lymphoma BM infiltration) were obtained through density gradient separation. BM MNCs were cultured in RPMI medium supplemented with 10% FBS and treated ex vivo with DMSO (control) or .1 nM E_2 for 24 hours. The effects of E_2 treatment on the expression levels of telomerase subunits genes (*TERT*, *TERC*, and *DKC1*) were assessed by quantitative PCR after 24 hours of incubation. E_2 was purchased from Selleckchem and DMSO from Sigma. RNA extraction was performed using the pure Link RNA mini kit (Invitrogen, Thermofisher, Waltham, Massachussets, USA). The RNA was quantified using "NanoDrop" and the quality verified running 200 ng RNA on a 1% agarose gel. Subsequently, 200 to 500 ng RNA was retrotranscribed using the Quanta VWR kit (Quantabio, Beverly, Massachussets, USA). For some samples RT minus was made to check the absence of genomic DNA. For gene expression analysis, 5 ng cDNA was amplified (in triplicate) in a reaction volume of 10 μL containing 5 μL TaqMan Fast Advanced Master Mix (Thermofisher) and .5 μL TaqMan Gene expression assay 20x (Thermofisher). Real-time PCR was carried out on the 7900HT Fast Real-Time PCR (Thermofisher), using a pre-PCR step of 20 seconds at 95°C, followed by 40 cycles of 1 second at 95°C and 20 seconds at 60°C. Samples were amplified with primers and probes for each target, and for all targets 1 NTC sample was run.

Raw data were analyzed with Biogazelle qbase plus software (Biogazelle, Zwijnaarde, Belgium), and the fold change was expressed as calibrated normalized relative quantity with standard error. Gapdh-hprt1 geometric mean was used for normalization. The entire process (extraction, retrotranscription, gene expression, and data analysis) was performed by the qPCR Service at Cogentech, Milan, Italy. Gene list and probe sequences are as follows: *TERT*, Hs 00972650_m1; *TERC*, Hs03454202_s1; *DKC1*, Hs00154737_m1; *GADPH*, Hs99999905_m1; *HPRT1*, Hs99999909_m1.

Statistical Analysis

Patient characteristics were summarized by percentages, median values, and ranges. Differences between groups were calculated with the Student *t*-test. Differences in proportions between groups were analyzed with the Fisher exact test. Box-plot graphs were used to show distributions of TL and age across different groups. Differences were considered significant at $P < .05$. Statistical analysis was performed with the statistical software package Prism 6.0 (GraphPad Software, San Diego, CA), and post hoc power calculations were performed using the G-Power software (version 3.1) (Dusseldorf University, Germany) with an alpha of .05.

RESULTS

Leukocyte TL in UCBT Subjects

TL was assessed on PB MNCs from 36 UCBT subjects and compared with a control group of cord blood units (n = 46) and with PB MNCs from age-matched healthy donors (n = 85). TL from UCBT subjects displayed a normal distribution, with a mean of 8698 bp (median, 8501 bp; range, 6521 to 11,960). In line with previous reports showing accelerated telomere shortening after allogeneic BMT, TL after UCBT was decreased compared with TL of control UCB samples, as shown in Figure 1A. Nevertheless, TL of UCBT recipients was significantly increased compared with PB MNCs from age-matched healthy donors (mean, 7396 bp; median, 7275 bp; range, 4375 to 11,108), as illustrated in Figure 1A. This difference was maintained when the analysis was restricted to adult subjects aged > 40 years (data not shown). Finally, there were no significant differences in TL according to timing of TL assessment after transplant, with analogous results in subjects assayed at ≤12 months and those at >12 months since UCBT, as illustrated in Figure 1B.

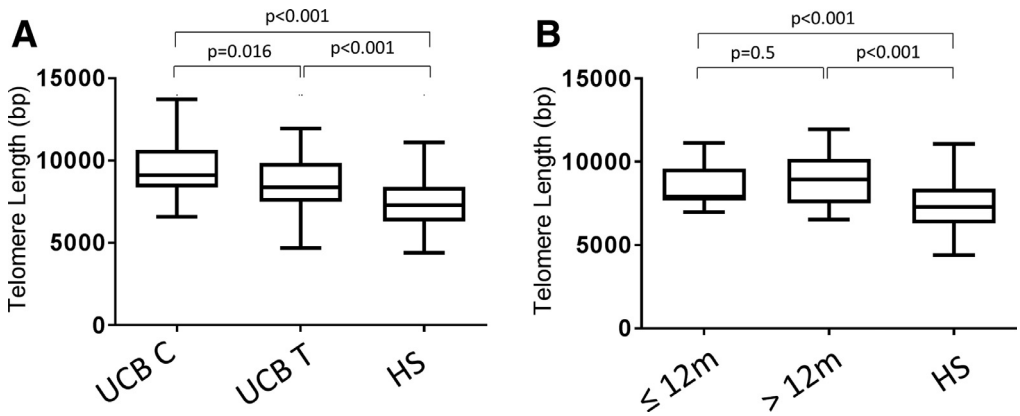

Figure 1. UCBT recipients maintain significantly longer telomeres compared with age-matched healthy control subjects. (A) Box plot graph showing TL of the UCBT recipient cohort (UCB T, n = 36) compared with a control group of UCBs (UCB C, n = 46) and age-matched healthy subjects (HS, n = 85). Differences between groups were calculated using the Student *t*-test. A $P < .05$ was considered as statistically significant. NS indicates not significant. Observed power (alpha = .05): UCB C versus UCB T, .76; UCB-T versus HS, .99; UCB C versus HS, .99. The box extends from the 25th to 75th percentiles, bars are min to max values. (B) Box plot graph showing TL of UCBT patients depending on the timing of TL measurement (>12 months, n = 21, versus ≤12 months, n = 15). Observed power (alpha = .05): ≤12 months versus >12 months, .16; ≤12 months versus HS, .85; >12 months versus HS, .93.

Factors Influencing TL in UCBT Recipients

To investigate any potential determinants of TL maintenance in UCBT subjects, several variables related to the recipient, type of graft, transplant procedure (including GVHD), disease status, and engraftment kinetics have been considered. All chronic GVHD events were recorded before TL testing. Regarding continuous variables, we first performed linear regression analyses and found no correlations between TL and recipient age (P = .28), number of MNCs or CD34$^+$ infused (P = .35 for MNCs, P = .82 for CD34$^+$), WBCs, platelet (PLT) counts, and hemoglobin levels at the time of TL assessment (P = .29 for WBCs, P = .82 for PLT counts, P = .09 for hemoglobin).

As detailed in Table 2, among factors related to recipient characteristics, recipient female gender was the only factor associated with long TL. Of note, recipient gender had no influence on WBCs, hemoglobin, or PLT counts (P = .19 for WBC, P = .53 for hemoglobin, P = .48 for PLTs). Among the investigated variables related to the graft and transplant procedure, only UCB gender correlated significantly with TL, with longer TL in subjects receiving male UCB graft compared with female UCB (Table 2). Thus, among all variables tested, only recipient and donor gender were found to have a significant impact on TL.

To further investigate TL dynamics according to recipient–donor gender, the 4 different combinations of donor–recipient gender were evaluated. As illustrated in Figure 2A, female UCBT recipients showed longer TL compared with male UCBT recipients, with the longest TL recorded in female recipients receiving male UCBs (mean, 10,063 bp; median, 9993 bp; range, 8381 to 11,960). Again, results were assessed according to the recipient age. When the analysis was restricted to subjects younger than 45 years, TL was significantly longer in female recipients receiving male UCB compared with those receiving female UCB; the difference was less pronounced when female recipients older than 45 years were separately assessed (Figure 2B). Furthermore, there was a trend toward longer telomeres in male UCBs transplanted into young (<45 years) female recipients compared with those transplanted into older recipients (≥45 years). No such trend was observed in female-to-female transplants (Figure 2B).

Table 2
Univariate Analysis of Factors Influencing Post-UCBT TL

Parameter	n	Median TL (range)	*P*	Median Recipient Age (range) (yr)	*P*
Recipient Gender					
Female	22	9423 (7128-11,960)		41 (8-69)	
Male	14	7589 (6521-9844)	.002	47 (5-64)	.367
Donor gender:					
Female	18	7855 (6521-10,282)		42 (8-64)	
Male	18	9088 (6630-11,960)	.04	42 (5-69)	.64
Body weight, kg					
≤58	18	8381 (6777 - 11,960)		38 (5-64)	
>58	18	8779 (6521-10,927)	.79	45 (17-69)	.06
Pre-UCBT disease status*					
CR	21	8115 (6630-11,149)		43 (8-64)	
<CR	13	9396 (6521-11,960)	.47	46 (10-69)	.15
MNCs infused, $\times 10^7$/kg					
≤3.35	18	8501 (6521-11,960)		46 (19-64)	
>3.35	18	8498 (6630-11,149)	.42	31 (5-69)	.01
CD34$^+$ cells infused, $\times 10^5$/kg					
≤1.4683	18	8787 (6521-11,960)		46 (5-64)	
>1.4683	18	8115 (4666-11,149	.86	36 (8-69)	.003
Type of UCBT					
Standard	23	8779 (6968-11,149)		40 (5-69)	
Intrabone	13	8381 (6521-11,960)	.44	45 (33-64)	.03
Acute GVHD (any grade)					
Yes	17	8381 (6521-11,149)		44 (8-64)	
No	19	8779 (6630-11,990)	.7	40 (5-69)	.87
Chronic GVHD (any grade)					
Yes	21	8387 (6521-11,960)		42 (9-69)	
No	15	8615 (6777-10,500)	.75	43 (5-64)	.32
WBCs/μL at TL assay					
≤6070	18	8248 (6521-10,464)		45 (8-64)	
>6070	18	9292 (7128-11,960)	.07	33 (5-69)	.07
Hb at TL assay, gr%					
≤12.4	18	7907 (6521-10,927)		42 (5-64)	
>12.4	18	9396 (6630-11,960)	.064	42 (10-69)	.84
PLTs/μL at TL assay:					
≤194,000	21	7907 (6521-11,149)		46 (8-64)	
>194,000	15	8615 (7128-11,960)	.18	34 (5-69)	.04

*Two patients were transplanted for non-neoplastic diseases (Fanconi anemia and thalassemia maior); therefore, the parameter disease status pretransplant is not applicable in these cases.

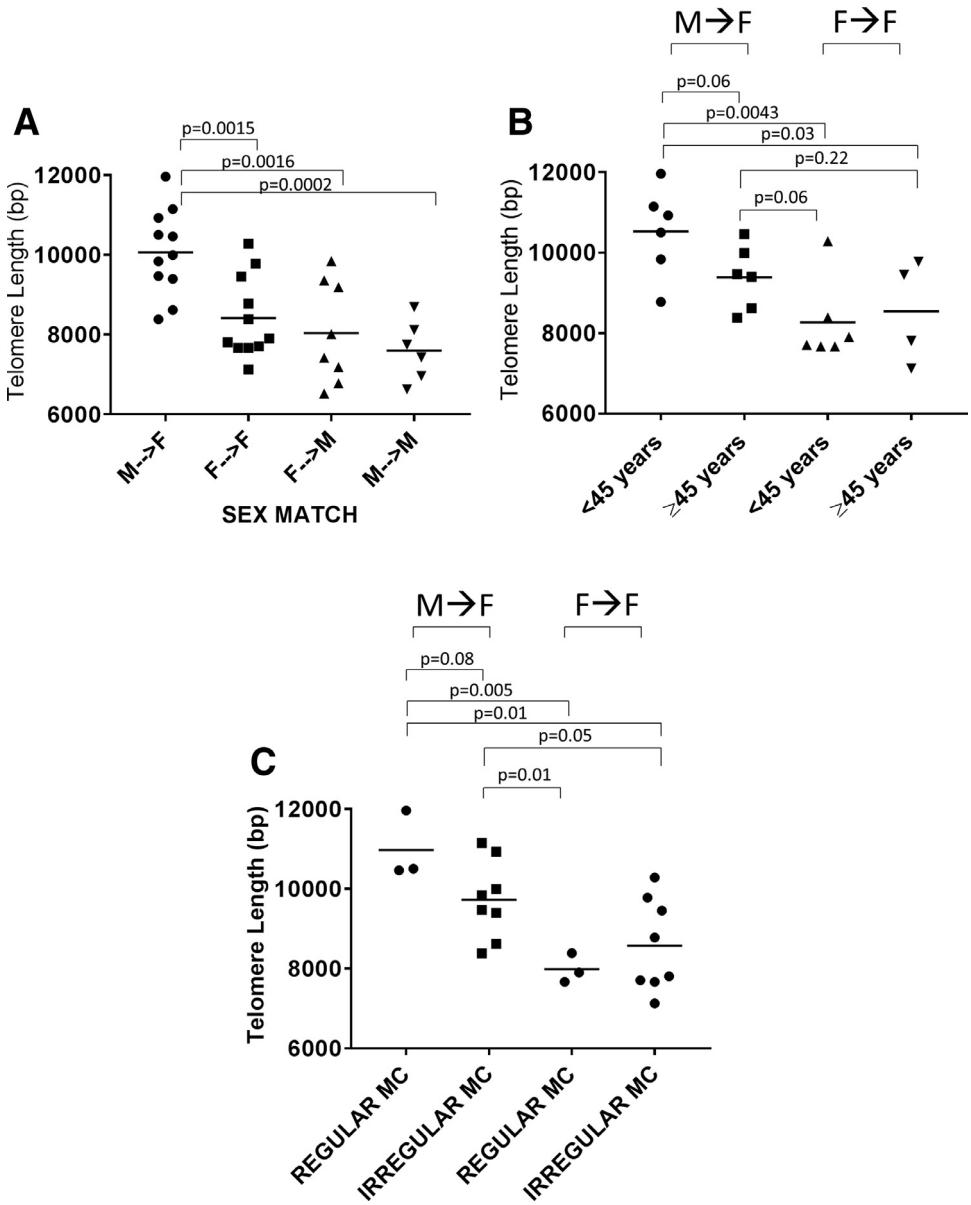

Figure 2. Determinants of TL in UCBT patient cohort. (A) Dot plot graphs showing longest TL in male to female (M→F) UCBT compared with all other subgroups (M→F n = 11; F→F n = 11; F→M n = 8; M→M n = 6). Differences between groups were calculated using the Student *t*-test. A $P < .05$ was considered as statistically significant. The bars represent the mean values. Observed power (alpha = .05): M→F versus F→F, .96; M→F versus F→M, .97; M→F versus M→M, .99. (B) Dot plot graph showing that TL was significantly longer in young (<45 years of age) female recipients receiving male UCB compared with those receiving female UCBs. Furthermore, there was a trend toward longer telomeres in young female compared with older female UCBT recipients receiving a male UCB graft (45 years is the median age of M→F transplant recipients). On the contrary, no such a trend in favor of younger recipients was observed in female to female transplants. (M→F < 45 n = 6; M→F ≥ 45 n = 6; F→F < 45 n = 6; F→F ≥ 45 n = 4). Observed power (alpha = .05): <45 M→F versus <45 F→F, .95; <45 M→F versus ≥45 M→F, .58; <45 M→F versus ≥45 F→F, .77; ≥45 M→F versus <45 F→F, .60; ≥45 M→F versus ≥45 F→F, .32. (C) Dot plot graphs showing longest TL in male UCB transplanted in women with regular menstrual cycles (MC). Among female recipients with regular MC (6/22), male UCBs had significantly longer telomeres compared with female UCBs. (M→F regular MC n = 3; M→F irregular MC n = 8; F→F regular MC n = 3; F→F irregular MC n = 8). Observed power (alpha = .05): M→F regular MC versus M→F irregular MC, .56; M→F regular MC versus F→F regular MC, .99; M→F regular MC versus F→F irregular MC, .90; M→F irregular MC versus F→F regular MC, .84; M→F irregular MC versus F→F irregular MC, .85.

Finally, in line with these data, the longest telomeres were observed in female recipients of male UCBs with regular menstrual cycles at the time of transplant, and among patients with regular menstrual cycles (n = 6, 28%), male UCBs displayed significantly longer telomeres compared with female UCBs (Figure 2C). The longer TL in UCBT recipients compared to healthy control subjects was maintained when the group with the longest TL (ie, female recipients receiving male UCB) was excluded from the analysis ($P = .03$, data not shown).

In Vitro Upregulation of Telomerase Subunits in Response to Estrogenic Stimulation in BM MNCs from Female and Male Donors

The observed longer TL in female recipients of male UCB suggests an increased sensitivity of male UCB cells to the female milieu. To verify this hypothesis, in vitro assays were designed to assess possible differences between female and male cells in response to E_2 exposure. To this aim, primary BM MNCs were treated in vitro with E_2 at picomolar concentrations (100 pM E_2, corresponding to the average E_2 serum

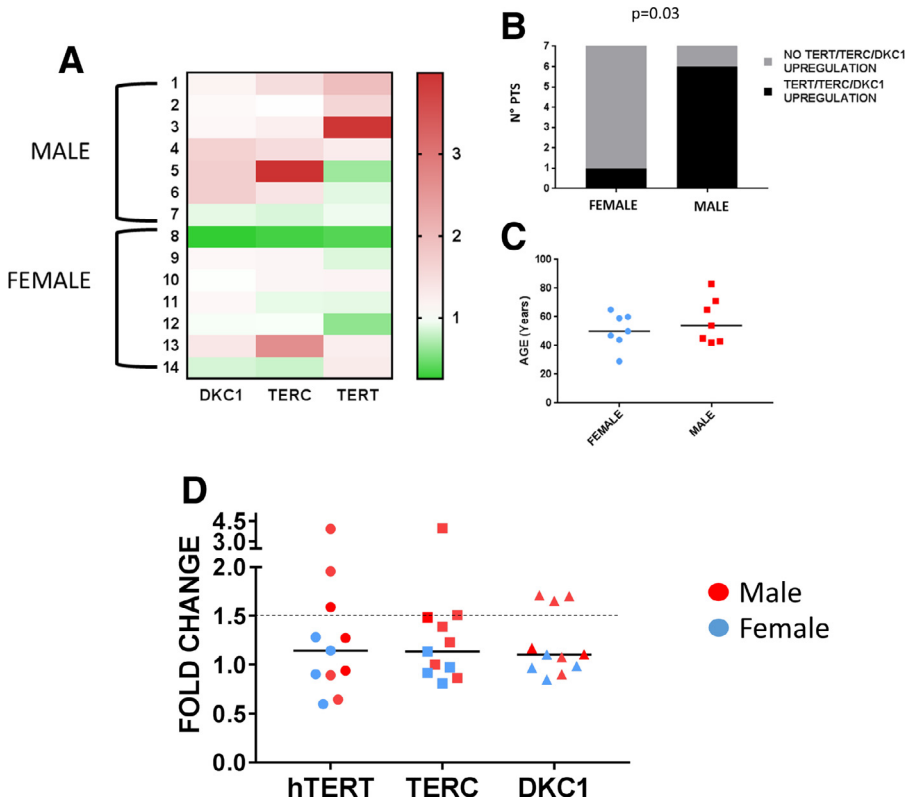

Figure 3. Ex vivo E_2 exposure upregulates telomerase subunits in male-derived bone marrow MNCs. (A) Heat map showing gene expression changes of telomerase subunits genes *TERT*, *TERC*, and *DKC1* in BM-derived MNCs from 14 subjects (7 men, 7 women) treated with E_2 .1 nM for 24 hours. Gene expression fold changes are shown in a colorimetric scale from green (low) to red (high). Primary BM MNCs from lymphoma patients undergoing initial staging procedures (who then resulted negative for lymphoma bone marrow infiltration) were obtained through density gradient separation, cultured in RPMI medium supplemented with 10% FBS, and treated ex vivo with DMSO (control) or .1 nM E_2 for 24 hours. mRNA was extracted and measured by TaqMan qPCR assays. (B) Bar graph showing the proportion of subjects upregulating at least 1 telomerase subunits (by a fold change $\geq$ 1.5) in male versus female subgroups. *P* value was calculated using the Fisher exact test. (C) Dot plot graph representing age in male (red dots) versus female (blue dots) subjects. Lines represent medians. (D) Dot plot graphs showing fold change of telomerase subunits mRNA levels after E_2 exposure (data shown in A) after postmenopausal female donors (n = 3) were excluded from the analysis. Significant upregulation of telomerase subunits (by a fold change $\geq$ 1.5) was observed exclusively in E_2-treated male-derived BM MNCs.

concentrations during the menstrual cycle). The effects of E_2 exposure was evaluated on the expression levels of telomerase subunits genes (*TERT*, *TERC*, and *DKC1*). As shown in the heat map represented in Figure 3A, male-derived primary BM MNCs treated with E_2 upregulated telomerase gene expression levels at a greater extent compared with their female counterparts. Notably, 6 of 7 male-derived primary BM MNCs displayed significant upregulation of at least 1 telomerase subunit, as compared with only 1 of 7 female-derived BM MNCs (Figure 3B). Interestingly, E_2 treatment resulted in upregulation of all 3 telomerase subunits (*TERT*, *TERC*, *DKC1*), with some subjects showing concomitant upregulation of multiple subunits (Figure 3A). There was no difference in age between male- and female-derived BM MNCs (Figure 3C). Excluding from the analysis postmenopausal women (n = 3, all > 50 years of age), significant upregulation of telomerase subunits was observed only in male-derived BM MNCs (Figure 3D).

DISCUSSION

In this study we evaluated telomere dynamics in the setting of UCBT, which provides the ideal setting to investigate the impact of extrinsic factors on TL, being a stem cell source characterized by very long telomeres not previously exposed to environmental factors. These unique features provide the optimal conditions to detect changes in telomere dynamics. The main findings of the present study are as follows. First, UCBT recipients display significantly longer telomeres in their MNCs compared with healthy age-matched control subjects. Second, the main determinant of TL in the post-UCBT setting is the recipient and donor gender, with female recipients receiving male UCB (M→F transplants) showing the longest telomeres. Third, ex vivo treatment with E_2 activated telomerase subunits predominantly in male-derived BM MNCs. The difference in TL between UCBT recipients and healthy age-matched control subjects in the present study was independent from the age of recipients, indicating that UCBT may restore a hematopoiesis that is not only healthy but also younger in terms of telomere signatures compared with that of the general healthy population. In other words, the replaced hematopoietic system after UCBT displays long telomeres even in an aged BM microenvironment. These findings are of particular interest given the reported association between leukocyte TL and longevity in the general population [22,23]. TL elongation after UCBT affords the possibility of using juvenile stem cells for hematopoietic rejuvenation.

The second finding of our study, mentioned above, is that extrinsic factors such as recipient and donor gender significantly affected TL in the post-UCBT setting. In this study post-UCBT TL was not affected by factors that may distinctly influence UCBT outcome, such as recipient body weight, number of MNCs and $CD34^+$ cells reinfused, quality of hematologic recovery and engraftments parameters, intrabone versus standard UCBT, disease status at transplant, and presence of GVHD. In fact, recipient and donor gender were the only factors that

significantly influenced postgraft TL. In our analysis, female recipients had a markedly longer leukocyte TL compared with men, with longest telomeres observed in male cells transplanted into female recipients. Although the data should be interpreted with caution given the small sample size, these observations suggest that the female milieu could favor the maintenance of long telomeres especially if engrafted with juvenile male stem cells. Accordingly, ex vivo treatment of BM-derived MNCs resulted in upregulation of telomerase subunits (*TERT*, *TERC*, *DKC1*), which was more prominent in male-derived than in female MNCs. The results indicate an increased sensitivity of male-derived hematopoietic cells to estrogen stimulation, providing mechanistic explanation of our observations in the UCBT setting. In line with this hypothesis, male UCBs transplanted into young female recipients displayed longer telomeres compared with those transplanted into older women, supporting the role of an intact hormonal female milieu to sustain telomere maintenance. On the other hand, recipient age had no impact on TL in female-to-female transplants. According to these observations, among patients with regular menstrual cycles, male UCBs showed significantly longer telomeres compared with female UCBs. These data suggest that an intact ovarian function in female recipients could determine a more potent and specific protection from telomere erosion in male UCBs.

We are aware of some intrinsic limitations of the present study, such as the small sample size and the absence of TL measurement before transplant, which could limit the ability to comment on post-transplant telomere dynamics and TL shortening. However, our in vitro data and the available literature on the impact of hormones on TL length strongly support the observations reported here.

In fact, several lines of evidence point to hormonal influence on TL maintenance. First, in line with our findings, estrogenic stimulation has been shown to increase *TERT* gene expression and activity in vitro and in animal studies [24–26]. Second, although estrogens have established antioxidant properties, on the contrary, testosterone does not prevent oxidative damage and has been linked to increased susceptibility to oxidative stress [14,27,28]. Third, male and female PB MNCs are characterized by different levels of estrogen receptors, with male monocytes showing higher expression of ER-α compared with monocytes derived from premenopausal females [29]. Notably, ER-α is believed to be the receptor through which sex hormones regulate hTERT activity [24]. Of note, according to our data, the analysis of TL in PB MNCs of dizygotic twins harboring a mixture of male- and female-derived cells (hematopoietic chimeras) demonstrated that in the female sibling male-derived PB MNCs had significantly longer telomeres compared with female cells and with their counterparts in the male sibling [30]. Furthermore, the in vitro responsiveness of human male-derived BM mesenchymal stem cells to E_2 has been previously demonstrated in a study showing increased proliferation of male-derived mesenchymal stem cells upon E_2 stimulation [31]. Moreover, a study showed a positive correlation between E_2 and dihydrotestosterone levels and TL in men, confirming that male PB-MNCs are responsive to sex hormone stimulation [32]. Finally, sex hormones have been demonstrated to counteract telomere erosion in human diseases characterized by telomere dysfunction [33].

A better knowledge of the mechanisms of TL regulation by potentially modifiable factors such as hormonal status could have broad therapeutic implications in the allogeneic BMT setting and beyond. The potential benefits from improved telomere maintenance in the allogeneic BMT setting has been highlighted by reports showing that donor TL predicts survival in patients undergoing allogeneic BMT in aplastic anemia [34], short TL correlates with the risk of life-threatening complications such as late BM failure and with infection-related deaths [35,36], and recipients of BMT showing extensive telomere attrition at 9 to 15 months post-transplant had low overall survival [37]. Although post-transplant complications, in particular GVHD, should be considered as modifiable factor potentially affecting TL, it should be noted that immunosuppressive drugs may cause direct telomere damage and shortening in leukocytes [38,39]. Similarly, we believe hormonal status could represent an actionable mechanism of TL regulation and a possible target for therapeutic intervention.

In addition to the allogeneic transplant setting, short TL is an established cancer risk factor [40–42]. Moreover, various anticancer agents, mainly chemotherapeutic drugs, can induce cell senescence along with telomere shortening [21,43–48]. This may increase the risk of second malignant neoplasm occurrence [49]. Hence, preemptive hormonal therapy might be considered as a potential treatment option to delay or mitigate telomere shortening in populations at risk of developing secondary neoplasms such as those previously exposed to drugs inducing telomere attrition or those harboring preneoplastic conditions associated with shortened telomere.

In conclusion, our results confirm the benefit of UCBT in terms of TL elongation and possible hematopoiesis rejuvenation. The observed impact of gender disparity between donor and recipient indicates a possible role of sex hormones in promoting telomere maintenance. Indeed, our observations both in vivo and in vitro provide the rationale for investigating hormonal therapy in the allogeneic BMT setting and as a strategy to counteract telomere shortening and aging-related diseases.

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