OPEN

Citation: Blood Cancer Journal (2014) **4**, e178; doi:10.1038/bcj.2013.75

www.nature.com/bcj

ORIGINAL ARTICLE

Prostaglandin E2 promotes survival of naive UCB T cells via the Wnt/β-catenin pathway and alters immune reconstitution after UCBT

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The outcome of umbilical cord blood transplantation (UCBT) is compromised by low hematopoietic stem cell (HSC) doses leading to prolonged time to engraftment, delayed immunological reconstitution and late memory T-cell skewing. Exposure of UCB to dimethyl-prostaglandin E2 (dmPGE2) increases HSC *in vivo*. We determined that exposure of UCB T lymphocytes to dmPGE2 modified Wnt signaling resulting in T cell factor (TCF)-mediated transcription. Wnt signaling upregulated interleukin (IL)-7R and IL-2Rβ, resulting in enhanced survival mediated by the homeostatic cytokines IL-7 and IL-15. dmPGE2 also induced components of the Wnt pathway and Wnt receptors, thereby priming UCB T cells to receive signals via Wnt ligands *in vivo*. We observed that the Wnt transcription factor *TCF7* and its target *EOMES* were elevated in the T cells of patients who received PGE2-treated UCBs. Consistent with the role of Wnt/β-catenin signaling to induce and maintain naive, memory precursors and long-lived central memory $CD8^+$ cells, these patients also had increased fractions of $CD8^+CD45RO^-CD62L^+$ plus $CD8^+CD45RO^+CD62L^+$ subsets encompassing these T-cell populations. These effects of the PGE2/Wnt/β-catenin axis may have significant implications for harnessing immunity in the context of UCBT, where impaired immune reconstitution is associated with late memory T-cell skewing.

Blood Cancer Journal (2014) **4,** e178; doi:10.1038/bcj.2013.75; published online 17 January 2014

Keywords: umbilical cord blood transplantation; immune reconstitution; prostaglandin E2; Wnt/β-catenin pathway

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is the only curative option for many patients with hematological malignancies, congenital disorders and bone marrow failure. Human leukocyte antigen-matched donor stem cells are preferred in order to avoid rejection and graft versus host disease. However, many patients do not have histocompatible donors.[1] Human umbilical cord blood (UCB) stem cells have emerged as an option for patients without suitable donors.[2] The use of UCB transplantation (UCBT) has steadily increased since the first transplant in 1988 to more than 30 000 recipients worldwide. In the United States, UCBT accounts for almost 20% of all HSCT annually.[3] As the absolute number of HSCs in the UCB unit is only ~10% of that present in traditional inoculum generated from adult donors, to compensate for the low HSC numbers, dUCBT (two umbilical cord blood units) are often used. However, engraftment remains suboptimal resulting in more post-transplant infectious complications than those observed in matched adult donor transplant recipients and even death. For these reasons, the development of methods and the identification of agents to increase UCB HSC homing, engraftment or HSC number is of significant therapeutic value.

It was previously demonstrated that PGE2 regulates vertebrate HSC induction and engraftment.[4] PGE2 was identified through a chemical genetic screen for modifiers of *runx1* expression within the zebrafish aorta-gonadal-mesonephros. Short-term (ST) *ex vivo* incubation with a stabilized derivative, 16,16-dimethyl-prostaglandin E2 (dmPGE2), enhanced the formation of stem cells and zebrafish marrow recovery following irradiation injury. PGE2 significantly enhanced embryonic stem cell hematopoietic colony formation and the frequency of both ST- and long-term repopulating HSC in the mouse bone marrow. Limiting dilution competitive transplantation analysis demonstrated a two- to fourfold increase in HSC number after short *ex vivo* PGE2 exposure, without creating an impact on multilineage hematopoietic differentiation or decreasing serial transplantation and self-renewal potential.[4,5] PGE2 functions through cyclic AMP (cAMP)-mediated regulation of the Wnt signaling pathway to control HSC homing, proliferation and survival.[5–7] On the basis of these findings, we performed a phase Ib pilot clinical trial of dUCBT, using one untreated and one *ex vivo* PGE2-treated UCB unit, to determine safety and engraftment parameters (http://www.clinicaltrials.gov (NCT00890500)). We demonstrated a significant reduction in the median time to engraftment compared with historic controls and skewing toward long-term hematopoiesis derived from the PGE2-treated UCB unit.[8]

Although these previous studies investigated the effects of ST *ex vivo* exposure of HSC to PGE2, the effect of this treatment on the T cells present in the UCB has not been examined. PGE2 has immunomodulatory effects and, when continuously present

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Received 5 December 2013; accepted 6 December 2013

during culture, alters the differentiation program of T cells by suppressing Th1 cell differentiation[9] and promoting development of Treg.[10] PGE2 can also mediate Th17 differentiation.[11] Although in other cell types PGE2 can induce direct Gsk3 phosphorylation and inactivation, thereby leading to stabilization of β-catenin and activation of T cell factor (TCF)/lymphoid enhancer-binding factor (LEF)-mediated gene activation,[7,12] whether PGE2 can modulate Wnt signaling in T cells has never been examined. Wnt/β-catenin signaling promotes quiescence and improves survival of $CD4^+$ T cells.[13,14] Wnt signaling controls the generation of long-lived memory $CD8^+$ T cells and highly potent stem cell memory $CD8^+$ T cells (T_{SCM}), which display naive immunophenotypic features and limited expansion but potent antigen-specific function.[13,15,16] Such effects of PGE2 might be of particular importance in UCBT where impaired immune reconstitution is characterized by T-cell skewing to a late effector $CD8^+$ phenotype.[17]

Here we examined the effects of ST exposure of UCB T cells to dmPGE2. We determined that transient *ex vivo* exposure of UCB T cells to PGE2 modified the Wnt signaling cascade through E-prostanoid (EP)2/EP4 receptors and cAMP-regulated phosphorylation of Gsk3, resulting in stabilization of β-catenin and TCF/LEF-mediated transcription. As a consequence, PGE2 induced an increase in interleukin (IL)-7Rα and IL-2Rβ mRNA and protein expression, enhanced survival mediated by the homeostatic cytokines IL-7 and IL-15 and protected UCB T cells against pro-apoptotic TCR-mediated signals. PGE2 also induced expression of Wnt pathway components and Wnt receptors, suggesting that *ex vivo* PGE2 treatment of the UCB might prime UCB T cells to receive Wnt/β-catenin signals *in vivo* after infusion into the UCBT recipients. Consistent with this hypothesis, using as a paradigm samples from four recipients of PGE2-treated UCBT recipients, we detected elevated expression of the transcription factors *TCF7* and *EOMES*, which are regulated by Wnt signaling. Thus, besides promoting HSC engraftment, *ex vivo* PGE2 treatment improves survival and immunological properties of UCB T cells in a Wnt-dependent manner.

MATERIALS AND METHODS

Cell isolation and culture

Mononuclear cells were isolated using Ficoll gradient centrifugation from fresh research umbilical cord blood units obtained from the Dana-Farber Cancer Institute (Boston, MA, USA). Umbilical cord blood units were collected according to local Institutional Review Board-approved protocols. Naive T cells were subsequently purified by negative selection using the Human Naive T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladhach, Germany). For T-cell activation and apoptosis induction, the tissue culture plates were coated overnight with 5 μg/ml of rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) at 4 °C and the following day they were washed three times and were incubated with anti-CD3 (0.7 μg/ml; OKT3, eBioscience, San Diego, CA, USA) for 1 h at room temperature. After three washes, cells were added to the plates and were incubated for the indicated time periods. Where indicated, soluble human recombinant IL-7 and IL-15 were added at a final concentration of 50 ng/ml. Short, transient PGE2 treatment was carried out by culturing naive T cells (5×10^6 cells per ml) with dmPGE2 (10 μM) for 2 h at 37 °C. Cells were subsequently gently washed one time with culture medium and used for the indicated studies.

cAMP measurement

Naive T cells were incubated with PGE2 for various time points (2–120 min). Cell pellets were collected and extraction was prepared by using Amersham cAMP Biotrak EIA system (GE Healthcare, Little Chalfont, UK). cAMP concentration in cell extracts was determined by enzyme immunoassay according to the manufacturer's instructions.

PKA assays

Naive T cells (1×10^6 cells per sample) were incubated with PGE2 (10 μM) for various intervals (5–60 min). Subsequently, cell pellets were collected and lysates were prepared and 0.02–1 μg of crude protein per sample was used. Protein kinase A (PKA) activity was determined using the PKA Kinase Activity Kit (Enzo Life Sciences, Plymouth Meeting, PA, USA). Relative kinase activity was measured using the formula provided by the manufacturer (average $\text{absorbance}_{\text{sample}}$ − average $\text{absorbance}_{\text{blank}}$)/quantity of crude protein used per sample). At least three different samples per condition were assessed and the experiment was repeated two times.

SDS-polyacrylamide gel electrophoresis and western blotting

Naive T cells were cultured with dmPGE2 for indicated time points and cell lysates were prepared and quantified as previously described.[18] Equal amounts of protein were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblot with antibodies specific for phospho-AKT, total AKT, phospho-GSK3β and total GSK3β. All antibodies were obtained from Cell Signaling Technologies (Danvers, MA, USA). Where indicated, densitometric analysis was performed and quantification of integrated density was assessed using the ImageJ 1.41o software (National Institutes of Health). Statistical significance was defined as a P-value < 0.05 and was calculated with the InStat software (GraphPad software, La Jolla, CA, USA).

Flow cytometry

The expression of β-catenin was assessed by intracellular staining with allophycocyanin-conjugated antibody against β-catenin followed by flow cytometry analysis (eBioscience). For the determination of cell death, cells were labeled with propidium iodide and Annexin-V (eBioscience), and were analyzed by flow cytometry (LSRII, Becton Dickinson, Franklin Lakes, NJ, USA).

TCF/LEF reporter assay

Naive T cells were transfected with TCF/LEF Cignal Reporter Assay (Qiagen, Valencia, CA, USA) by nucleofection (Lonza, Cologne, Germany). Four hours after transfection, the cells were collected and cultured with PGE2 for 16 or 20 h. The luciferase assay was performed by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following the manufacturer's protocol for developing assay.

Real-time PCR

Naive T cells were cultured with PGE2 (10 μM) at 37 °C for the indicated time intervals. Where indicated, naive T cells were incubated with 6-bromoindirubin-3′-oxime (BIO), a selective Gsk inhibitor or with *N*-methylated BIO, an inactive analog of BIO (R&D Systems/TOCRIS, Minneapolis, MN, USA) or with the older generation Gsk3 inhibitor TWS119 (EMD4Biosciences, San Diego, CA, USA). Total RNA extraction was prepared with the RNeasy Mini Kit from Qiagen according to the manufacturer's instructions. RNA was subjected to quantitative PCR (qPCR) analysis for the target genes *LEF1*, *JUN*, *EOMES*, *RUNX1*, *TCF7*, *CREM, c-MYC*, *CTNNB1*, *FZD1*, *FZD9, LRP5, LRP6, DV1, DV3 and AXIN2*. The FAM-conjugated primers for all target genes as well as the TaqMan One-Step reverse transcriptase-PCR Master Mix reagents (Life technologies, Grand Island, NY, USA) and the VIC-TAMRA (Life technologies)-conjugated 18S RNA housekeeping gene control primers were from Applied Biosystems/Roche, Branchburg, NJ, USA. The reaction was performed in an AB 7000 qPCR machine from Applied Biosystems. For assessment of EP receptors, previously described primers[6] were obtained from IDT (Coralville, IA, USA).

Clinical trial

This research protocol was approved by the Institutional Review Board of the Dana-Farber/Harvard Cancer Center. Written informed consent was obtained from all patients for the correlative laboratory study of immune reconstitution before enrollment and participation. The trial was prospectively registered at http://www.clinicaltrials.gov (NCT00890500). Detailed patient characteristics and clinical outcome of the entire clinical trial are reported elsewhere.[8] Samples collected from patients who received one untreated and one PGE2-treated UCB unit (PGE2-UCBT) were compared with samples from patients who received two untreated UCB units (control-UCBT).

Statistical analysis

In vitro assay, densitometry data and qPCR data were compared with unpaired *t*-test. Analysis of variance was performed to compare the

expression levels of *EOMES* and *TCF7* measured at days 0, 30, 60 and 100 after transplantation. A two-sided *P*-value of <0.05 was considered statistically significant.

RESULTS

PGE2 modulates Wnt/β-catenin signaling in UCB T cells

In adult T cells, PGE2 is known to signal via EP2 and EP4 receptors.[11] To determine whether UCB T cells were capable of transmitting PGE2-mediated signals, we examined expression of EP receptors. Naive UCB T cells expressed EP2 and more prominently EP4 receptor, and lacked expression of EP1 and EP3 receptors (Figures 1a and b). dmPGE2 induced a robust elevation of intracellular cAMP within 2 min of incubation, which gradually declined over 2 h of treatment (Figure 1c). In other cell types, via EP2 and EP4 receptors, PGE2 regulates intracellular phosphorylation through cAMP and the downstream effector kinases PKA and PI3K/Akt.[12] To determine whether EP2 and EP4 receptors were capable of transmitting signals in UCB T cells, we examined the activation of PKA and PI3K/Akt pathways. dmPGE2 induced activation of PKA (Figure 1d) and PI3K, leading to phosphorylation of the PI3K target Akt (Figures 1e and f).

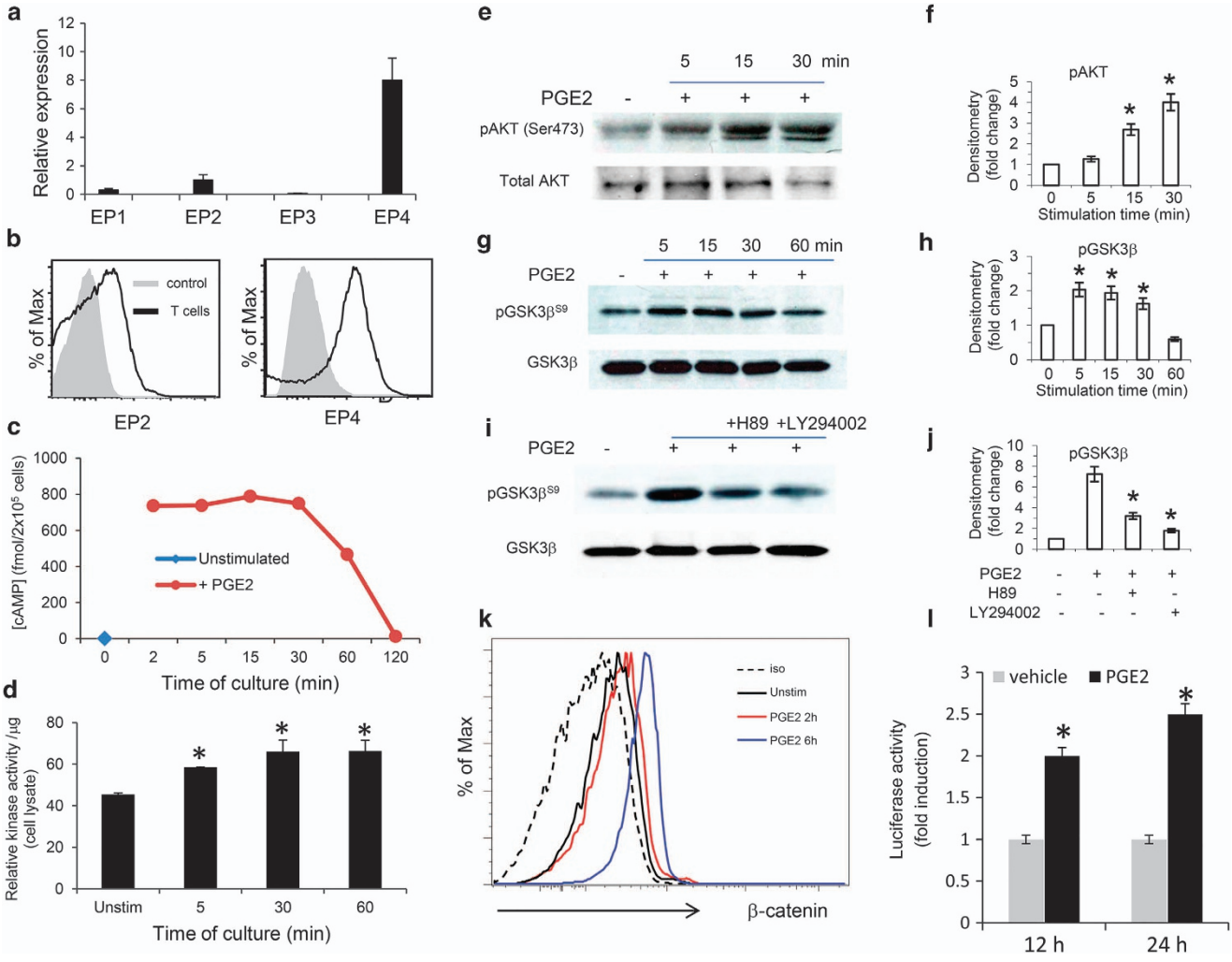

Figure 1. PGE2 induces activation of Wnt/β-catenin signaling. (**a**, **b**) Naive T cells isolated from UCB and expression of *EP1*, *EP2*, *EP3* and *EP4* genes was examined by qPCR (**a**). Data were normalized to β-actin. Results are representative of three independent assessments. Expression of EP2 and EP4 receptors in naive UCB T cells was also analyzed by flow cytometry (**b**). (**c**–**j**) Naive T cells isolated from UCB were incubated with dmPGE2 (10 μM) for indicated time intervals. Levels of intracellular cAMP were determined by enzyme immunoassay (**c**). The data shown are representative of two performed experiments. (**d**) PKA activity was measured by enzyme-linked immunosorbent assay using PKA kinase activity kit. Relative kinase activity was calculated as described in Materials and Methods. Data are means ± s.d. of triplicate from one experiment. PKA kinase activity was significantly increased after incubation of T cells with PGE2 compared with that in untreated T cells (*$P<0.05$). The data represent two independent experiments. (**e**, **g**) WT cells were incubated with PGE2 for the indicated time intervals, and phosphorylation of Akt and GSK3β was assessed by immunoblot with phospho-specific antibodies. (**i**) T cells were treated for 15 min with PGE2 in the presence or absence of inhibitors for PKA (H-89) or PI3K (LY294002) and phosphorylation of GSK3β was examined. (**f**, **h**, **j**) Densitometric analysis of the abundance of each indicated phosphorylated protein was normalized to that of total protein, and was expressed as fold-change relative to mean values before stimulation (defined as 1). Data are presented as the mean ± s.e.m. *$P<0.05$, $n=4$ experiments. (**k**) Accumulation of β-catenin incubation of naive T cells with PGE2 was assessed by intracellular staining and flow cytometry. Similar effects were observed by the use of the Gsk3 inhibitor TWS119 (Supplementary Figure 1a). The data shown were generated from one experiment and are representative of three independent experiments. (**l**) Naive T cells were transfected with TCF/LEF cignal reporter. After 4 h, cells were treated with 10 μM PGE2 or vehicle alone. A dual-luciferase assay was performed 12 and 24 h after incubation of T cells with PGE2, and reporter activity values were expressed as arbitrary units using a Renilla reporter for internal normalization. Reporter activity was significantly increased in T cells that were treated with PGE2 (*$P<0.05$).

Signaling via EP2/EP4 receptors in HSC and via transfected EP2 and EP4 receptors in HEK cells can induce inactivating phosphorylation of Gsk3 via PKA and PI3K/Akt,[7,12] leading to Gsk3 inactivation and stabilization of β-catenin. The levels of β-catenin are tightly controlled by the destruction complex, consisting of axin, GSK3, CK1 and allophycocyanin. In the absence of Wnt signaling, β-catenin is phosphorylated at N-terminal residues by GSK3β and targeted for ubiquitination/degradation.[19] In contrast, C-terminal phosphorylation of β-catenin stabilizes the protein by inhibiting destruction.[20] Destruction complex assembly can be blocked by phosphorylation of Gsk3β at Ser9, which can be mediated by PKA and Akt.[21] Consequently, β-catenin is accumulated and translocated into the nucleus to regulate the expression of Wnt target genes. As our studies showed that PGE2 induced activation of PKA and Akt in UCB T cells, we examined the phosphorylation of GSK3β at Ser9. dmPGE2 induced rapid and robust phosphorylation of GSK3β at Ser9 (Figures 1g and h). To dissect whether PKA or Akt was responsible for the phosphorylation of GSK3β, we used the PKA inhibitor H-89 and the PI3K inhibitor LY294002. There was a reduction in PGE2-mediated phosphorylation of GSK3β when the cells were treated with either inhibitors, but inhibition was more prominent when the PI3K inhibitor LY294002 was used (Figure 1j). Together with the higher expression of EP4 than EP2 (Figures 1a and b), this finding indicates that in UCB T cells PGE2 signals predominantly via the EP4 receptor, which selectively activates the PI3K/Akt pathway, in contrast to the EP2 receptor that preferentially activates PKA.[12] As a result of the blockade of destruction complex assembly by the inactivating phosphorylation of Gsk3β at Ser9, expression of β-catenin was increased in UCB T cells treated with dmPGE2 (Figure 1k).

The functional consequence of β-catenin accumulation is to bind to TCF/LEF transcription factors, leading to transcriptional activation of Wnt-responsive genes. To evaluate the potential of PGE2 in modulating Wnt-mediated gene transcription in UCB T cells, we performed transient transfections with the TCF/LEF-specific reporter. Assessment of reporter activity showed a significant increase of luciferase activity in UCB T cells incubated with PGE2 compared with vehicle control (Figure 1l). Of note, the increase of Wnt-mediated transactivation induced by PGE2 in UCB T cells was of moderate magnitude, similar to previous observations of PGE2-mediated effects on Wnt-regulated transcriptional activity in HSC.[7] Our data suggest that PGE2 modifies Wnt/β-catenin signaling in UCB T cells by increasing the accumulation of β-catenin and promoting TCF/LEF-mediated transcriptional activity.

PGE2 induces Wnt target genes in UCB T cells

To determine whether our finding that dmPGE2-induced TCF/LEF-mediated reporter activity in UCB T cells was biologically relevant, we assessed expression of genes known to be regulated downstream of canonical Wnt signaling. Real-time quantitative reverse transcriptase-PCR showed that the expression of *LEF1*, *JUN, EOMES*, *RUNX1* and *TCF7*, all of which have been identified as Wnt/β-catenin-regulated targets,[7,15,16,22,23] were increased in UCB T cells by dmPGE2 incubation (Figure 2a). PGE2 also upregulated expression of CREM in UCB T cells, a gene previously identified as a Wnt target induced by Wnt3A treatment of thymocytes.[22] In contrast, *c-MYC*, a Wnt target gene upregulated in human colorectal cancer[24] and previously identified to be induced by PGE2 in HSC,[7] was not upregulated in UCB T cells. Instead, PGE2 downregulated *c-MYC* expression in UCB T cells (Figure 2b), suggesting that PGE2-mediated Wnt response genes are cell type-specific. As in HSC, PGE2 also regulates expression of its own receptors,[6] we examined whether PGE2 incubation might alter expression of EP receptors in UCB T cells. PGE2 treatment had no effect on EP2 (data not shown) but significantly augmented expression of EP4 (Figure 2a).

To verify that the effect of PGE2 on the upregulation of these genes was mediated via transcriptional mechanisms downstream of β-catenin, we used the iCRT3, -5 and -14 inhibitor, which blocks β-catenin-responsive transcription by binding to β-catenin and disrupting the interaction between β-catenin and TCF1.[25] Assessment of three representative Wnt target genes showed that incubation of UCB T cells with iCRT inhibited induction *of LEF1, JUN* and *EOMES* by dmPGE2 (Figure 2c). These results provide evidence that PGE2 induced expression of these genes via modulation of the β-catenin-mediated transcriptional activity in UCB T cells.

PGE2 enhanced the anti-apoptotic effect of the homeostatic cytokines IL-7 and IL-15

Previous studies showed that Wnt signaling in T cells prevents the downregulation of IL-7Rα-chain (CD127), which occurs during T-cell activation, and preserves high levels of IL-7Rα expression.[16,23] IL-2Rβ chain (CD122), a shared subunit between IL-2 and IL-15 receptors with an essential role in IL-15-mediated anti-apoptotic function, is also upregulated via the Wnt/TCF pathway.[15] IL-7 and IL-15 promote survival and homeostasis of naive T cells, development and survival of memory T cells, and enhance immune reconstitution after HSCTs.[26–29] To evaluate whether PGE2 pretreatment of UCB T cells might have an effect on T-cell survival mediated via these homeostatic cytokines, first we examined the effects of dmPGE2 on the expression of IL-7Rα and IL-2Rβ. After ST treatment with dmPGE2 or vehicle control, UCB T cells were stimulated via TCR/CD3 and expression of IL-7Rα and IL-2Rβ was assessed. PGE2 treatment significantly diminished the downregulation of IL-7Rα induced by TCR stimulation (Figure 3a). Although no apparent difference in the expression of IL-15Rα was observed, IL-2Rβ was increased (Figure 3a). Notably, both IL-7Rα and IL-2Rβ mRNA were induced by PGE2 treatment alone and their induction was abrogated by incubation of T cells with iCRT (Figure 3b), indicating that PGE2 induced expression of these genes by modulating β-catenin-mediated transcriptional activity. To further investigate whether IL-7Rα and IL-2Rβ were Wnt target genes, we used BIO a specific inhibitor of Gsk3β.[30] Bio-acetoxime treatment resulted in robust increase of both IL-7Rα and IL-2Rβ mRNA. In contrast, treatment with *N*-methylated BIO, an inactive analog of BIO-acetoxime did not alter expression of either IL-7Rα or IL-2Rβ mRNA (Figure 3b). These results provide evidence that IL-7Rα and IL-2Rβ are Wnt target genes and their expression is upregulated by PGE2 in UCB T cells. As a consequence of this effect, when combined with TCR-mediated stimulation, PGE2 prevented the decrease of IL-7Rα mRNA and augmented the upregulation of IL-2Rβ mRNA mediated by TCR signals (Figure 3c).

To determine the biological implications of these findings, we examined whether PGE2 might enhance the pro-survival effects of IL-7 and IL-15 and protect UCB T cells, which are highly prone to TCR-mediated apoptosis.[31] After prior treatment either with dmPGE2 or vehicle control, UCB T cells were cultured with plate-bound OKT3 monoclonal antibody and apoptosis was assessed by Annexin-V and propidium iodide staining. TCR-mediated stimulation of control-treated T cells resulted in a significant degree of apoptosis (Figure 3d). Culture of PGE2-treated T cells under the same conditions induced a slight but reproducible decrease of apoptosis, which did not reach statistical significance. Addition of IL-7 or IL-15 during TCR-mediated stimulation improved survival in both control- and PGE2-treated UCB T cells. However, during culture with either IL-7 or IL-15, apoptosis of PGE2-treated UCB T cells was significantly decreased as compared with control-treated UCB T cells (Figure 3d).

To further analyze the effects of PGE2 on the apoptotic machinery activated by TCR/CD3, we evaluated the levels of active, cleaved caspase-3. TCR-mediated stimulation of

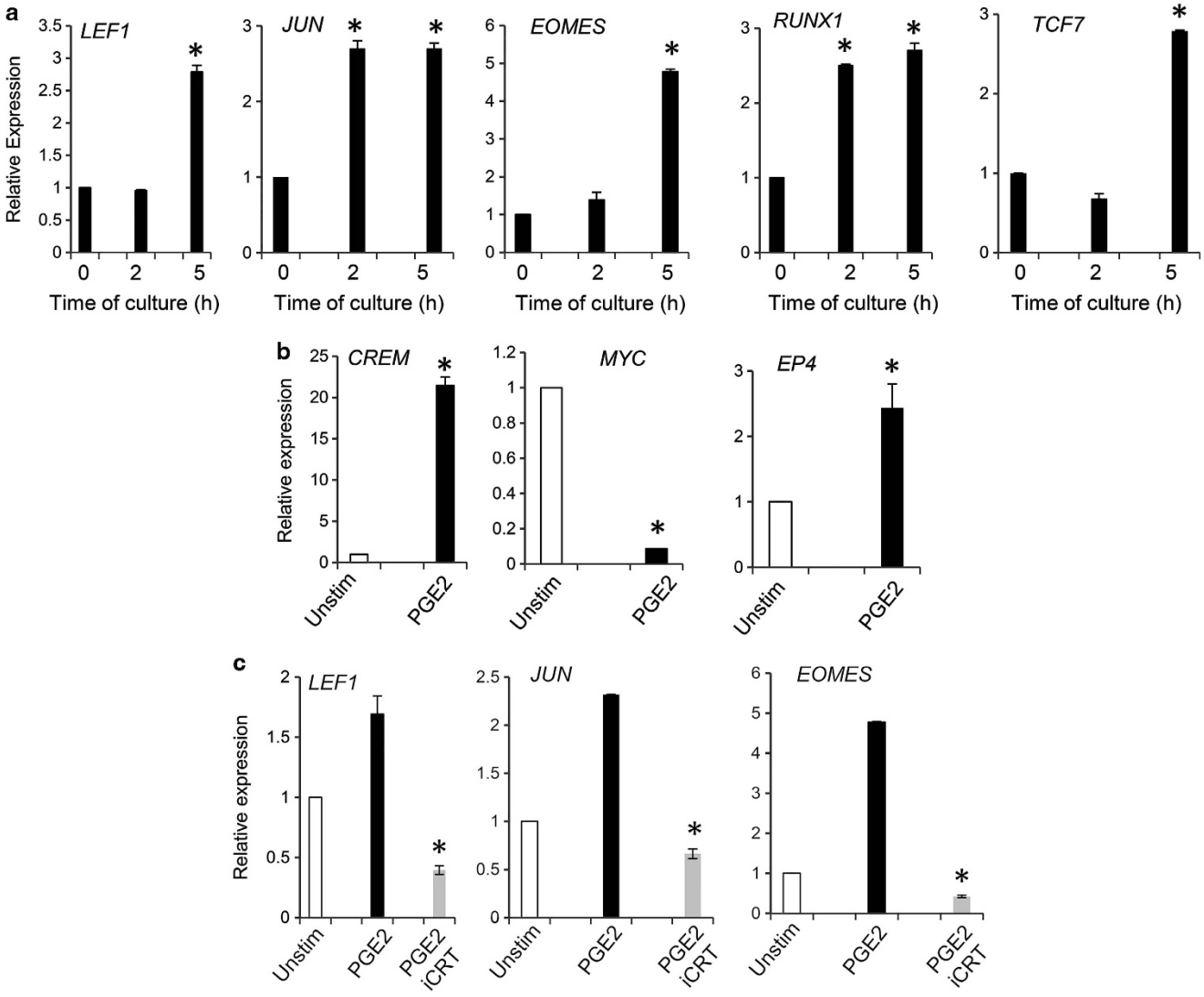

Figure 2. PGE2 regulates expression of Wnt/β-catenin target genes. (**a**) Naive T cells isolated from UCB were cultured with dmPGE2 (10 μM) for the indicated time intervals and RNA was isolated and expression of *LEF, JUN, EOMES, RUNX1* and *TCF7* was assessed by quantitative reverse transcriptase-PCR. (**b**) Assessment of *CREM*, *c-MYC* and EP4 mRNA was performed at isolation and after 5 h of culture with dmPGE2. The expression of the indicated genes was significantly altered after PGE2 incubation, compared with untreated T cells (*$P<0.05$). No changes in the expression in any of the tested genes were detected upon culture with vehicle alone for the same time intervals (data not shown). The effects of the Gsk3 inhibitor TWS119 on two representative Wnt target genes were tested in parallel (Supplementary Figures 1b). (**c**) After culture for 5 h with PGE2 in the presence of vehicle (PGE2) or iCRT (60 μg/ml) (PGE2 + iCRT), expression of *LEF, JUN* and *EOMES* was assessed as in **a**. Data are presented as mean ± s.d. The expression of the indicated genes was significantly reduced after addition of iCRT compared with incubation with PGE2 with vehicle alone (*$P<0.05$). The data represent two independent experiments.

control-treated T cells resulted in caspase-3 activation that was only slightly reduced by PGE2 pretreatment (Figure 3e). Addition of IL-7 or IL-15 during TCR-mediated stimulation reduced caspase-3 activation in both control-treated and PGE2-treated UCB T cells. Under these conditions, there was a 50% decrease of caspase-3-positive fractions in PGE2-pretreated T cells compared with control-treated UCB T cells (Figure 3e). These results indicate that PGE2 pretreatment protects UCB T cells under pro-apoptotic conditions and suggest that T cells of the PGE2-treated UCB may display survival advantage *in vivo* after infusion to the UCBT recipients.

PGE2 induces components of the Wnt/β-catenin pathway and Wnt receptors in UCB T cells

The Wnt pathway regulates not only the expression of Wnt target genes but also the expression of Wnt pathway signaling components and Wnt receptors.[15,22] As our studies showed that PGE2 induced modulation of Wnt/β-catenin signaling and gene transcription in UCB T cells, we examined whether genes encoding for components of the Wnt pathway and/or Wnt receptors were also affected. Using real-time qPCR, we determined that PGE2 induced a significant increase of the Wnt pathway component *CTNNB1,* the Wnt receptors *FZD1*, *FZD9* and the co-receptor *LRP6* (Figure 4). PGE2 also upregulated *DVL* molecules, which connect the Fzd-Lrp receptor and the β-catenin destruction complexes. These molecular effects were specific and selective because the members of these gene families were not globally affected by PGE2 in UCB T cells (Figure 4). These results show that short transient incubation of UCB T cells with PGE2 induces regulatory components of the Wnt pathway and Wnt receptors and suggest that upon *ex vivo* PGE2 treatment, UCB T cells might be primed to receive enhanced Wnt/β-catenin-mediated signaling through Wnt ligands *in vivo* after infusion to the UCBT recipients.

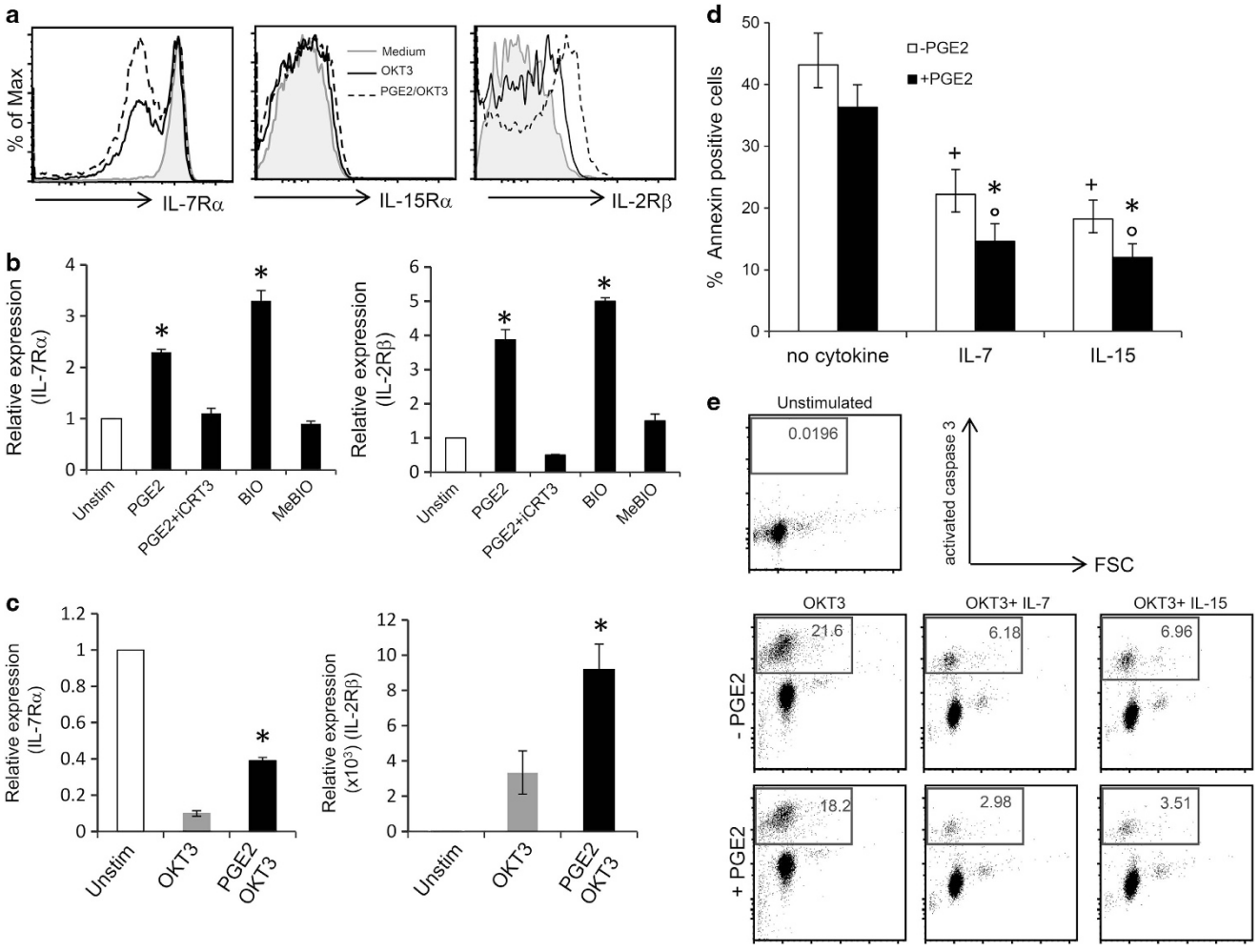

Figure 3. PGE2 incubation results in increased expression of IL-7Rα and IL-2Rβ and enhances the anti-apoptotic effects mediated by IL-7 and IL-15 in UCB T cells. (**a**) Naive T cells treated with PGE2 or vehicle control for 2 h were stimulated with plate-bound anti-CD3 (OKT3, 0.7 μg/ml). Twenty-four hours after the initiation of culture, expression of IL-7Rα, IL-15Rα and IL-2Rβ was examined by flow cytometry. Naive T cells were cultured with either PGE2, PGE2 plus iCRT3, BIO or *N*-methylated BIO (MeBIO) for 2 h (**b**) or with OKT3 in the presence of vehicle or PGE2 (**c**), and expression of IL7Rα and IL-2Rβ mRNA was determined by qPCR. Data are means ± s.d. of duplicates. PGE2 significantly upregulated expression of IL-7Rα and IL-2Rβ mRNA (*$P<0.05$, $n=2$). (**d**, **e**) Naive UCB T cells were treated with either vehicle or PGE2 for 2 h and were subsequently stimulated with plate-bound anti-CD3 (OKT3, 0.7 μg/ml) in the presence of IL-7 (50 ng/ml) or IL-15 (50 ng/ml) or absence of these cytokines. After 24 h of culture, the cells were collected and apoptosis was measured by Annexin-V staining **d**. Data are means ± s.d. of two independent experiments. Addition of IL-7 or IL-15 significantly prevented TCR-mediated apoptosis of control-treated cells ($^{+}P<0.05$) or PGE2-treated UCB T cells ($^{o}P<0.05$) compared with the relevant cultures without addition of these cytokines. During culture with either IL-7 or IL-15, treatment with PGE2 significantly reduced apoptosis compared with control-treated UCB T cells (*$P<0.05$). (**e**) Activated caspase-3 was detected by staining cells with antibody specifically against cleaved caspase-3 followed by flow cytometry analysis. Treatment of naive T cells with PGE2 for 2 h did not induce an inhibitory effect on T-cell proliferation or cytokine production upon subsequent stimulation (Supplementary Figures 2a and b).

Increased expression of Wnt-regulated transcription factors and altered fractions of CD8^{+} T-cell subsets in PGE2-UCBT recipients

As our *in vitro* studies suggested that transient *ex vivo* incubation with PGE2 might prime UCB T cells to receive enhanced signals through Wnt ligands, we sought to determine whether evidence of amplified Wnt signaling might be detectable *in vivo*. We used as a paradigm T cells from patients from a phase Ib pilot clinical trial of dUCBT who received one untreated and one *ex vivo* PGE2-treated UCB unit (thereafter named recipients of PGE2-UCBT). These patients developed complete T-cell chimerism from the PGE2-treated UCB unit as early as day +13.[8] T cells transferred with the graft are the dominant source of circulating T lymphocytes before thymic regeneration, which occurs after 3 months from UCBT.[17,32] Thus, in recipients of PGE2-UCBT, T cells of the UCB unit that was exposed to PGE2 *ex vivo* consist the dominant circulating T-cell population in the first 3 months after UCBT. Using real-time qPCR, we assessed expression of Wnt-regulated hallmark transcripts *TCF7* and *EOMES* in the peripheral blood lymphocytes of UCBT recipients. As compared with recipients of control-UCBT, recipients of PGE2-UCBT had increased expression of *TCF7* and *EOMES* at all the time points tested (Figures 5a and b). These differences were unlikely to be due to random individual variability because assessment of these genes in healthy controls showed comparable expression (data not shown). We performed analysis of variance on *TCF7* and *EOMES* with repeated measures at days 0 (pre-transplant), 30, 60 and 100, and we found that both *TCF7* and *EOMES* were significantly higher in the PGE2-UCBT group ($P<0.001$) compared with the control-UCBT group. The interaction terms between *TCF7* or *EOMES* and days were also significant ($P<0.001$) indicating that

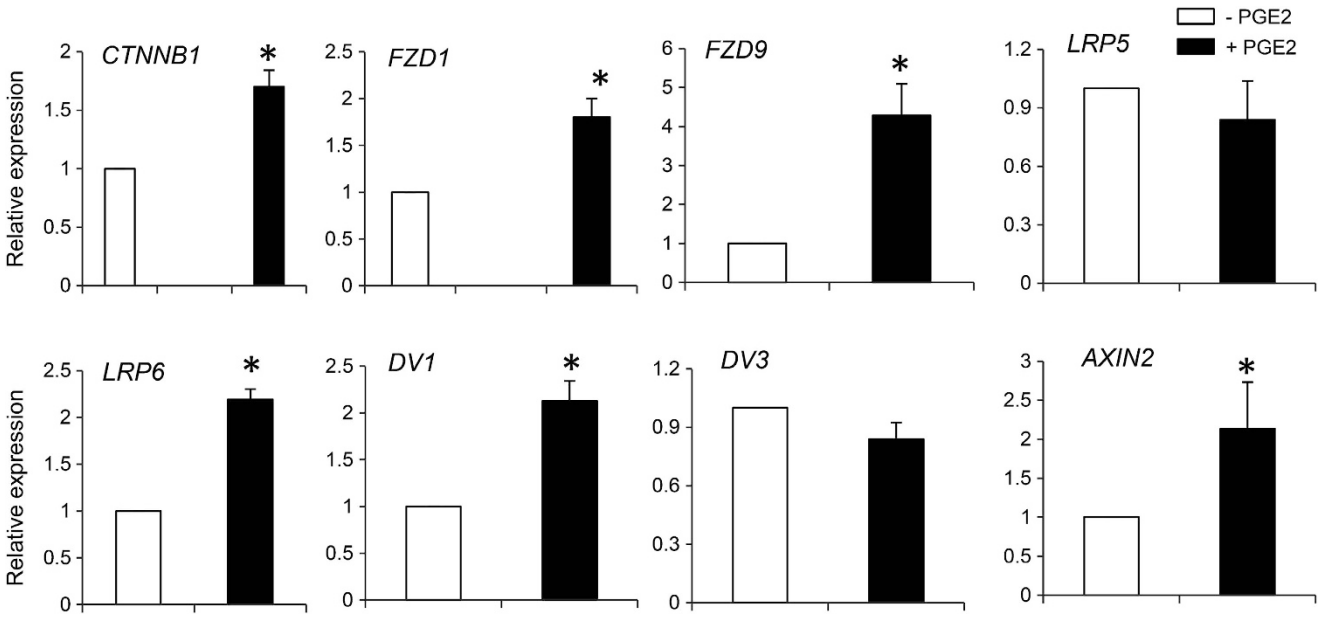

Figure 4. PGE2 regulates expression of the Wnt-β/catenin pathway components in UCB T cells. Naive UCB T cells were cultured with dmPGE2 for 2 h and expression of the indicated genes was assessed by qPCR. Data are means ± s.d. and represent two independent experiments (*$P<0.05$). *AXIN2*, a universal Wnt target gene pathway,[52] was used to verify Wnt pathway signaling. Increase of *AXIN2* is shown at 5 h of culture because shorter time intervals of incubation resulted in only slight increases that did not reach statistical significance.

expression of *TCF7* and *EOMES* in the PGE2-UCBT cohort increased over time, whereas this pattern was not seen in the control-UCBT group.

A major defect contributing to the impaired immune reconstitution after UCBT is the paucity of naive and central memory $CD8^+$ T cells and the skewing to a late effector $CD8^+$ T-cell subset.[17] This mechanism is a significant cause of the impaired immune function after UCBT because naive and the central memory $CD8^+$ subsets are more potent regulators of protective immunity than effector memory and effector $CD8^+$ cells.[33–35] Wnt/β-catenin signaling arrests $CD8^+$ T-effector cell terminal differentiation, preserves naive T cells and induces the generation and maintenance of central memory $CD8^+$ T cells.[13,15,16,23,36,37] Notably, *SELL* encoding for CD62L, which is expressed specifically in naive and central memory $CD8^+$ T cells, is a direct target of the Wnt transcription factor TCF1 (encoded by *TCF7*).[15] For these reasons, we examined whether *ex vivo* PGE2 treatment of T cells present in the UCB might prevent the terminal differentiation of $CD8^+$ T cells induced *in vivo* after UCBT[17] and might augment the naive and central memory $CD8^+$ T-cell fractions. We used the established combination of CD45RO and CD62L markers to identify naive ($CD45RO^-CD62L^+$), central memory ($CD45RO^+CD62L^+$), effector memory ($CD45RO^+CD62L^-$) and late effector memory ($CD45RO^-CD62L^-$) $CD8^+$ T-cell subsets. We analyzed proportional distribution of these $CD8^+$ T-cell fractions before transplantation and during the first 100 days after UCBT. At baseline, the $CD8^+$ compartment was characterized by low levels of naive and paucity of central memory cell fractions in both groups (Figure 5c). After UCBT, in control recipients, the bulk proportion of $CD8^+$ T cells expressed predominantly markers of effector memory and late effector differentiation, and suppressed $CD62L^+$ subsets, encompassing $CD8^+CD45RO^-CD62L^+$ and $CD8^+CD45RO^+CD62L^+$ cells (Figures 5c and d), consistent with previous observations.[17] In contrast, PGE2-UCBT recipients had higher $CD8^+CD45RO^-CD62L^+$ and $CD8^+CD45RO^+CD62L^+$ cell fractions (Figures 5c and d). Although this difference did not reach statistical significance because of the small sample size ($P=0.07$), there was a clear trend for a higher proportion of the combined $CD8^+CD45RO^-CD62L^+$ plus $CD8^+CD45RO^+CD62L^+$ fractions in PGE2-UCBT recipients (Figure 5d). These findings do not intend to make clinically relevant conclusions because our study was not designed to address clinically relevant questions related to Wnt-mediated effects *in vivo*. However, these findings can serve as a paradigm, which supports the hypothesis that short, transient exposure to PGE2 may alter the properties of UCB T cells after UCBT in a Wnt-dependent manner.

DISCUSSION

Our studies showed that short incubation with dmPGE2 altered the molecular and functional properties of human UCB T cells by inducing a robust increase of cAMP via EP2 and EP4 receptors and activating a cascade of signaling events, which resulted in inactivating phosphorylation of GSK3β, stabilization of β-catenin and TCF/LEF-mediated gene expression. PGE2 incubation induced increased expression of IL-7Rα (CD127) and IL-2Rβ (CD122) resulting in enhanced anti-apoptotic effects of IL-7 and IL-15 cytokines and a survival advantage of PGE2-treated UCB T cells under pro-apoptotic conditions. PGE2 also upregulated Wnt pathway components and Wnt receptors, suggesting that *ex vivo* treatment with PGE2 could prime UCB T cells to receive Wnt-mediated signaling *in vivo* after transfer to the UCBT recipients.

Wnt signaling in mouse and human T cells limits effector T-cell differentiation and induces the generation of $CD8^+$ T_{SCM} cells, which display immunophenotypic features of naive T cells and 'stemness' properties, characterized by quiescence, self-renewal and multipotency. These cells give rise to central memory, effector memory and effector $CD8^+$ T cells in response to antigen re-exposure and mediate protective immunity.[16,38] Studies in mouse models have shown that Wnt and its downstream target TCF7 have a mandatory role for the generation and maintenance of long-lived protective memory $CD8^+$ cells, whereas in the absence of Wnt/TCF7 signals there is a dominance of short-lived terminally differentiated effector $CD8^+$ T cells that fail to mount protective immune responses.[13,15,37,39] Samples from our pilot clinical trial that was designed to study safety and engraftment

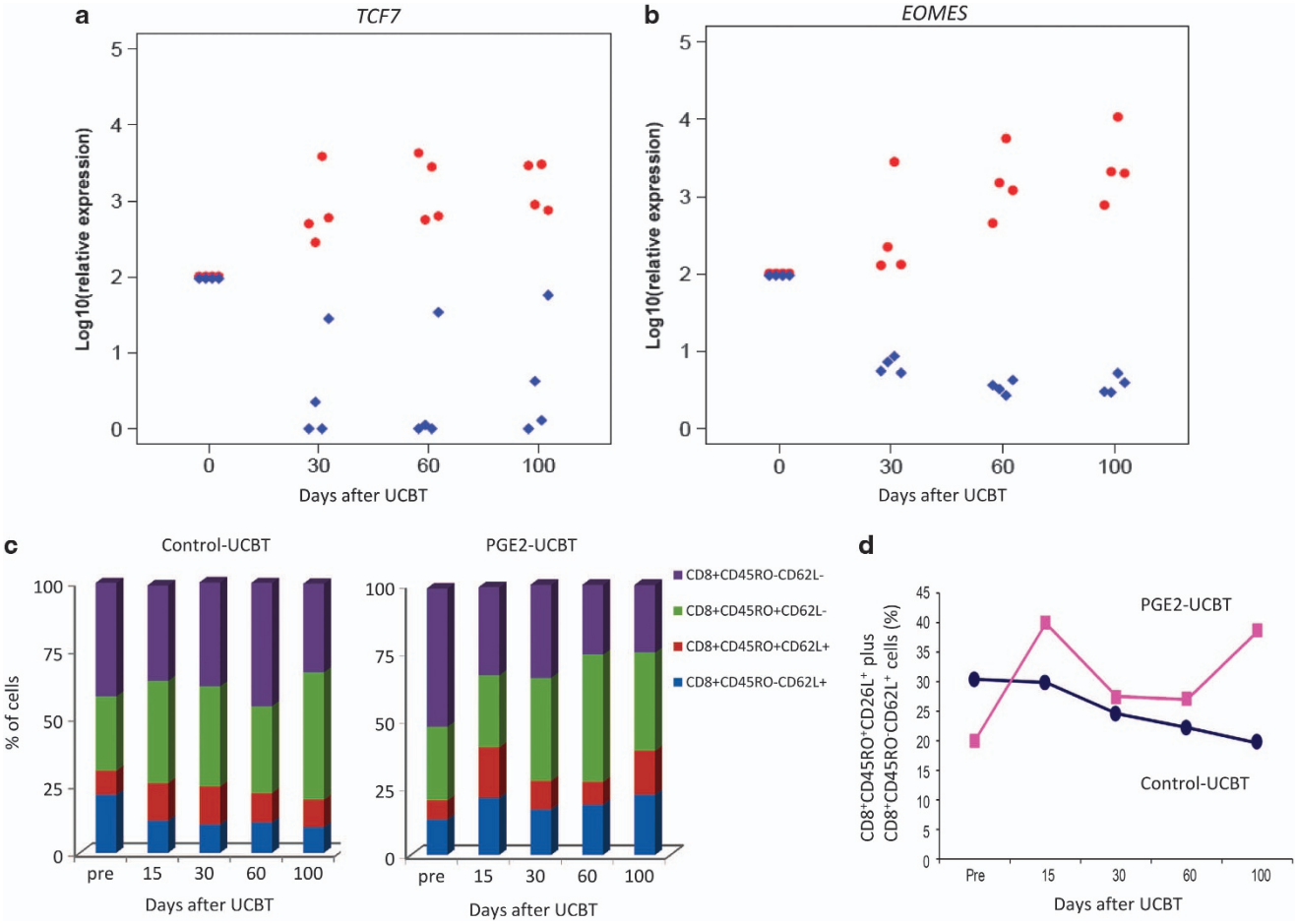

Figure 5. Increased expression of functional central memory genes and altered CD8+ T-cell reconstitution in PGE2-UCBT recipients. (**a**, **b**) Peripheral blood T lymphocytes from four control-UCBT recipients (blue diamonds) and four PGE_2-UCBT recipients (red closed circles) were analyzed for expression of *TCF7* and *EOMES* by real-time quantitative reverse transcriptase-PCR. Relative change of gene expression was calculated with respect to levels on day 0, considered as 100 (100%). We performed analysis of variance on log10-transformed *TCF7* and *EOMES* with repeated measures at days 0 (pre-transplantation), 30, 60 and 100, and we found that both *EOMES* and *TCF7* were significantly higher in the PGE2-UCBT group ($P<0.001$) compared with the control-UCBT group. A similar pattern was observed in five additional patients in the PGE2-UCBT group but because samples were not available for all the time points of follow-up, these patients were not included in the plots. (**c**) The relative proportions of the four indicated $CD8^+$ T-cell subsets were assessed using three-color flow cytometry. (**d**) The combined $CD8^+CD45RO^-CD62L^+$ plus $CD8^+CD45RO^+CD62L^+$ T-cell fractions, encompassing naive, memory precursors and central memory cells, displayed distinct kinetics of reconstitution and a clear trend for higher values in PGE2-UCBT recipients versus control-UCBT recipients ($P=0.07$). Results represent mean values of 9 control-UCBT recipients and 12 PGE2-UCBT recipients.

parameters after *ex vivo* PGE2 treatment of a UCB unit provided a supportive translational paradigm to our *in vitro* studies on the molecular and biochemical effects of PGE2 on UCB T cells. Consistent with our *in vitro* data that PGE2 induced components of the Wnt pathway and Wnt receptors, our correlative studies indicated that PGE2 enhanced and sustained the activation of the Wnt/β-catenin/TCF axis in the lymphocytes of PGE2-UCBT recipients, as determined by the increase in the expression of the Wnt target genes *TCF7* and *EOMES*. These findings are consistent with the conclusion that PGE2 primed T cells to receive enhanced Wnt-mediated signals through Wnt ligands *in vivo*. Whereas the identity and source of Wnt ligands that signal to T cells *in vivo* remain to be elucidated, it has been shown that macrophages and *in vitro*-differentiated dendritic cells can produce Wnt7b and Wnt5a, respectively,[40,41] and that multiple Wnt transcripts including Wnt1, 2B, 4, 5A and 8B are detected in vascular endothelial cells.[42] Thus, PGE2-treated T cells expressing high levels of Wnt receptors may be capable of repeatedly receiving signals mediated via Wnt ligands during circulation and encounter with dendritic cells. This hypothesis can explain the persistent elevation of *TCF7* and *EOMES* observed in the T cells of PGE2-UCBT recipients. Interestingly, PGE2-UCBT recipients had higher factions of $CD8^+CD45RO^-CD62L^+$ and $CD8^+CD45RO^+CD62L^+$ T cells. Although these two cell subsets were traditionally considered to represent naive and central memory $CD8^+$ T cells, respectively, it was recently determined that the newly identified T_{SCM} population shares immunophenotypic properties with both these cell subsets.[38] Thus, these cell populations in PGE2-UCBT recipients may also contain T_{SCM} $CD8^+$ cells.

T_{SCM} cells were initially described as a very small fraction within the $CD45RO^-CD45RA^+CCR7^+CD62L^+IL\text{-}7R\alpha^+$ naive cell population, which is characterized by elevated expression of IL-2Rβ (CD122) and CD95.[38] In spite of their naive-like immunophenotype, T_{SCM} cells show functional properties of memory T cells and can mount potent responses against viral and tumor antigens.[16,38] It was recently reported that besides CD45RA and other surface markers of naive cells including CD62L, CCR7 and IL7Rα, T_{SCM} cells also express CD45RO and represent a distinct $CD8^+$ T-cell subset, intermediate between naive and central memory cells.[43] Although our pilot correlative studies in

the samples of PGE2-UCBT recipients were not designed to assess T_{SCM} cells, the molecular and immunophenotype properties induced by PGE2 in naive UCB T cells *in vitro* resemble these features of T_{SCM} cells. We determined that PGE2 upregulated IL-7Rα and IL-2Rβ and enhanced IL-7- and IL-15-mediated effects in UCB T cells. As besides Wnt signaling,[16,38] IL-7 and IL-15 can also instruct the generation of T_{SCM} cells,[43] our findings suggest that *ex vivo* PGE2 treatment of UCB T cells might promote the generation of T_{SCM} cells *in vivo* by enhancing Wnt/β-catenin-mediated signals and IL-7/IL-15-mediated signals. Thus, PGE2 might promote the formation and maintenance of T_{SCM}, similar to HSCs. Future studies will focus specifically on the effects of PGE2 on the quantitative and qualitative profiles of T_{SCM} cells.

Similar to previously identified effects of PGE2 on $CD34^+$ HSC, in UCB T cells PGE2 upregulated expression of *RUNX1*, a molecule with a critical role in T-cell polarization and functional differentiation. In the presence of Foxp3, Runx1 has an indispensable role in the generation and function of Treg, whereas in the presence of RORγt, Runx1 is required for the generation of Th17 cells.[44] This consequence of PGE2-mediated signals might have significant implications on the *in vivo* differentiation program and the plasticity of UCB T cells, which in the context of distinct microenvironmental factors might be capable of undergoing differentiation to Treg or to Th17 cells.[45,46] This finding might also account for the divergent previous reports regarding the ability of PGE2 to induce generation of Treg and Th17 cells.[10,11] Through this mechanism, PGE2 might also instruct the differentiation of IL-17-producing $CD8^+$ T cells, which have potent antitumor properties.[47] Of note, our studies showed that short incubation with PGE2 upregulated EP4 suggesting that *ex vivo* PGE2-treated T cells are primed to receive enhanced *in vivo* signaling mediated by PGE2, which is elevated after HSCT.[48]

Our studies showed that PGE2 treatment upregulated the expression of IL-2Rβ (CD122), prevented the downregulation of IL-7Rα (CD127) in response to TCR stimulation and enhanced the anti-apoptotic effects of IL-7 and IL-15 upon TCR-mediated apoptosis of UCB T cells. Serum IL-7 and IL-15 levels increase from the baseline upon lymphopenia induced by conditioning chemotherapy, promote T-cell survival and enhance immune reconstitution after HSCT.[29] Thus, PGE2-treated UCB may have survival advantage over untreated T cells in the presence of IL-7 and IL-15 after infusion to the UCBT recipient. Consistent with this hypothesis, in our phase I clinical trial, complete T-cell chimerism from PGE2-treated UCB was detected as early as day +13.[8] Although single-donor chimerism is generally documented within 1–3 months after double UCBT, the mechanisms of UCB unit dominance have not yet been fully unraveled.[49,50] Recently, it was determined that UCB unit dominance in leukocyte subsets is established within 18 days after transplantation and that chimerism in $CD4^+$, $CD8^+$ and natural killer T cells at day +11 is predictive of the ultimate UCB unit dominance.[51] It is tempting to speculate that PGE2-mediated survival advantage of UCB T cells might be responsible for the early T-cell chimerism observed in PGE2-UCBT recipients. This event might also have an indispensable role in the PGE2-UCB unit dominance, which is further promoted by the effects of PGE2 on the homing and survival of long-term repopulating HSC.[6,7] Further studies are required to determine how PGE2 treatment individually affects $CD4^+$, $CD8^+$ and natural killer T-cell subsets and whether it alters their alloreactive and cytolytic properties, which might be involved in a presumed alloreactive graft versus graft rejection *in vivo*.[51]

By improving HSC engraftment while favoring the dominance of naive plus central memory $CD8^+$ T cells, which provide protective immunity,[33–35] *ex vivo* PGE2 treatment of the UCB might improve the outcome of UCBT, where delayed engraftment and impaired immunity due to late effector T-cell skewing[17] are serious causes of morbidity and mortality. These effects of the PGE2/Wnt/β-catenin axis in human T cells may also have significant implications for harnessing immune memory in the context of tumor-specific and pathogen-specific immunity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by NIH Grants HL107997-01, R56AI43552, RO1CA183605-01, U54HL081030, T32AI07549, the Leukemia and Lymphoma Society Translational Research Program TRP 6222-11 and by the HHV6 Foundation Award.

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