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Dppa5 Improves Hematopoietic Stem Cell Activity by Reducing Endoplasmic Reticulum Stress

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SUMMARY

Developmental pluripotency-associated 5 (Dppa5) is an RNA binding protein highly expressed in undifferentiated pluripotent stem cells. Here, we demonstrate that Dppa5 is a regulator of hematopoietic stem cells (HSCs) that critically governs reconstitution capacity after bone marrow transplantation. Ectopic expression of Dppa5 followed by in vitro culture robustly increased HSC reconstitution levels through suppression of endoplasmic reticulum (ER) stress and apoptosis. Remarkably, a chemical chaperone that decreases ER stress in HSCs also increases HSC engraftment. Conversely, knockdown of Dppa5 impaired the long-term reconstitution ability of HSCs due to elevated ER stress levels, suggesting that ER stress regulation is physiologically important for proper HSC function in vivo. Thus, Dppa5 represents a pivotal connection between ER stress regulation and stem cell properties in HSCs. The findings also demonstrate that protein quality control is critical for the maintenance, survival, and function of HSCs in vivo and ex vivo.

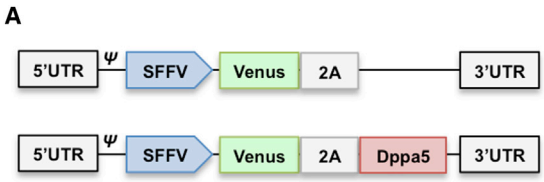
INTRODUCTION

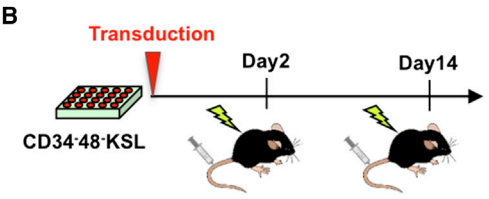
Hematopoietic stem cells (HSCs) have limited ability to proliferate in culture in contrast to their strong capacity in vivo (Morrison and Kimble, 2006). Attempts have been made to expand HSCs for clinical use by adding specific hematopoietic growth factors in culture or by induction of HSC-specific regulators. The exploration to find critical HSC regulators has identified a limited number of genes that act as major effectors on HSC self-renewal in vitro, e.g., Angptls and HoxB4 (Antonchuk et al., 2002; Zhang et al., 2006).

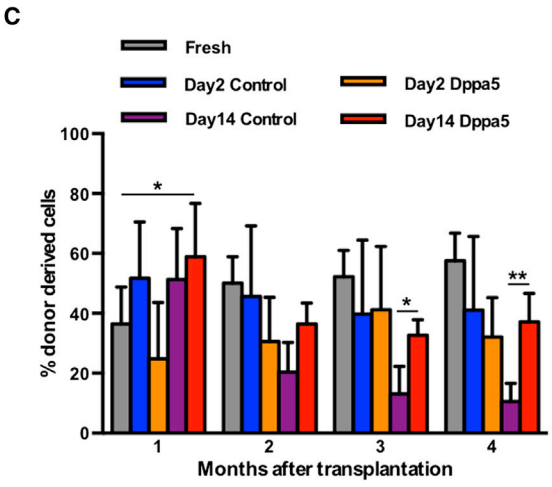
Under steady state, HSCs are maintained dormant within the stem cell niche in the bone marrow (BM), minimizing the risk for damage (Ema and Suda, 2012). HSCs are particularly found in areas of low oxygen (hypoxic niche), where glycolysis is used as a main energy provision, and oxidative stress can be minimized (Kubota et al., 2008; Simsek et al., 2010; Takubo et al., 2010; Miharada et al., 2011). Accumulation of reactive oxygen species (ROS) severely impairs the long-term reconstitution capacity of HSCs through p38 mitogen-activated protein kinase (MAPK), and inhibition of this pathway effectively maintains HSC function in vitro (Ito et al., 2006; Baudet et al., 2012). Thus, expansion or maintenance of HSCs may need elimination of stress factors generated in actively growing cells and in ex vivo culture.

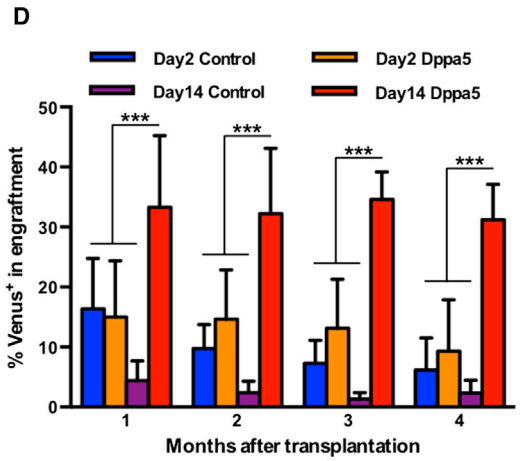
Endoplasmic reticulum (ER) stress is triggered by multiple stress factors, including viral infections, ROS, and proliferative signals (Walter and Ron, 2011). Higher protein synthesis upon enhanced cell proliferation and abundant protein production/secretion leads to accumulation of unfolded and mis-folded proteins, which is a major cause of the ER stress (Luo and Lee, 2013). To recover from this, the ER stress response, also called the unfolded protein response (UPR), is activated as a cell-defensive mechanism (Walter and Ron, 2011). The ER stress response is a multistep reaction governed by multiple signaling pathways, inducing both survival and apoptotic signals for maintaining cell and tissue homeostasis (Luo and Lee, 2013). Resolving or resisting ER stress is critical for cells, in particular to highly proliferating cells. It is known that many tumor cells including leukemia have highly upregulated expression of ER stress chaperones, e.g., heat shock proteins (hsps), offering increased resistance to the ER stress, and deprivation of ER stress chaperones is critical for tumor growth and progression (Luo and Lee, 2013). In addition, during embryogenesis, avoidance of ER stress induction is crucial because deletion of GRP78 in embryos leads to an increase in ER stress level resulting in peri-implantation lethality (Luo et al., 2006). Thus, a large series of studies depicts the importance of ER stress regulation in actively growing cells; however, the effect of ER stress in HSCs during reconstitution and in homeostasis has remained unclear.

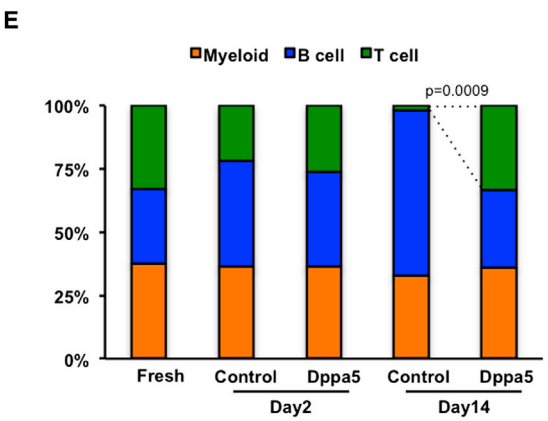
Developmental pluripotency-associated 5 (Dppa5/Esg1) is an RNA binding protein and is known for its high expression in undifferentiated pluripotent stem cells (Kim et al., 2005; Amano et al., 2006; Tanaka et al., 2006). In this study, we demonstrate that Dppa5 regulates HSCs by diminishing ER stress. Overexpression of Dppa5 robustly increases the reconstitution capacity of HSCs after in vitro culture, and ER stress levels and subsequent apoptotic signals are significantly reduced upon ectopic Dppa5 expression. Conversely, downregulation of Dppa5 expression abolishes long-term reconstitution by HSCs due to increased apoptosis caused by elevated ER stress levels. Moreover, we demonstrate that reduction of ER stress levels using a chemical chaperone is a potential treatment to support HSC reconstitution capacity. Thus, Dppa5 provides pivotal connections between protein quality control and stem cell properties in HSCs.

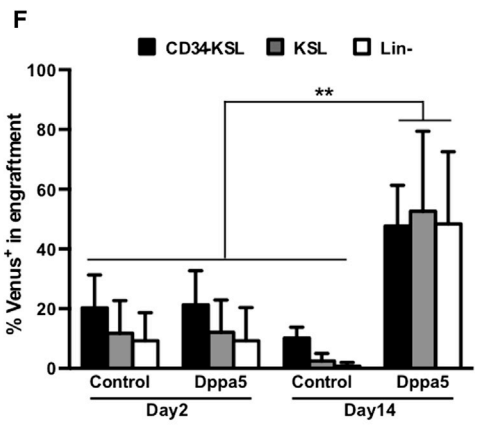


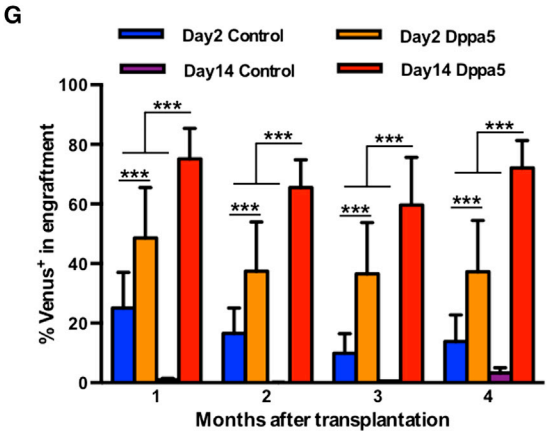












H

		Equivalent cell number	Number of positive mice	HSC frequency	Abs. HSC number	Fold change compared with initial HSC
Exp.1	Control	20	1/3	1:70465	4.27	0.0142 (4.27/300)
		10	0/3			
		1	0/3			
	Dppa5	20	6/6	1:4477	145.19	0.4840 (145.19/300)
		10	6/6			
		1	1/5			
Exp.2	Control	20	1/3	1:81182	5.00	0.0250 (5.00/200)
		10	0/3			
		1	0/4			
	Dppa5	20	4/4	1:5162	108.49	0.5425 (108.49/200)
		10	5/5			
		1	2/5			
Exp.3	Control	10	3/3	1:21875	17.19	0.0860 (17.19/200)
		5	1/3			
		1	0/4			
	Dppa5	10	4/4	1:1628	323.10	1.6155 (323.10/200)
		5	5/5			
		1	4/5			

(legend on next page)

RESULTS

Dppa5 Enhances Long-Term Reconstitution of HSCs

To evaluate the functionality of Dppa5 in long-term HSCs, competitive reconstitution analysis was performed with lentiviral overexpression of Dppa5 (Figure 1A). $CD34^{-}48^{-}$KSL cells transduced with the control (Venus alone) or Dppa5 lentiviral vector (Dppa5) were transplanted into lethally irradiated mice in a competitive setting on day 2 and day 14 after culture to test HSC expansion and activation ex vivo (Figure 1B). Monthly peripheral blood (PB) analyses showed that cells overexpressing Dppa5 for 14 days in vitro had significantly higher reconstitution than control-transduced cells, whereas no significant difference was seen in the recipients engrafted with Dppa5-transduced cells cultured for 2 days (Figures 1C and 1D). No significant difference was detected between the engraftment of fresh cells and cells cultured for 2 days with the control vector, indicating that there was no major negative impact on the engraftment efficiency from culturing the cells for 2 days with the control vector. Mice transplanted with Dppa5-overexpressing HSCs cultured for 14 days showed significantly higher frequency of $Venus^{+}$ cells in the engraftment, indicating that the reconstitution advantage of Dppa5-overexpressing cells is generated during the in vitro culture (Figure 1D). Importantly, the ratio of Venus-expressing cells in the total engraftment was stable in Dppa5-overexpressing cells over time, suggesting that they were otherwise governed by physiological HSC regulatory pathways (Figure 1D). Dppa5-overexpressing cells also maintained multilineage potential (myeloid lineage, B cell, and T cell), even after ex vivo culture for 14 days, whereas T cell production capacity was severely impaired in control cells (Figure 1E). However, the improvement in reconstitution by Dppa5 in the primary recipients was not higher than the reconstitution generated by fresh cells (Figure 1C). The reconstitution advantage of Dppa5 was also detected in the BM, showing robust and significantly increased reconstitution of transduced, immunophenotypic hematopoietic stem and progenitor cells (Figure 1F). To further evaluate the effects of Dppa5 on long-term reconstitution, total BM cells from selected primary recipient mice were transplanted into secondary recipients. The advantage of the reconstitution ability of 14-day cultured Dppa5-overexpressing cells was further enhanced upon secondary transplantation, whereas the Venus control cells failed to reconstitute (Figure 1G). In addition, 2-day cultured Dppa5-overexpressing cells also showed significant advantage compared to control cells upon secondary transplantation (Figure 1G). This clearly suggests a long-term competitive advantage of Dppa5-overexpressing cells. To ask whether the number of HSCs was increased, limiting dilution transplantation analysis was performed. After 14 days in culture, transduced cells were transplanted with different cell doses. The findings showed no clear increase in the HSC number compared to the starting cell number, although the frequency of HSCs was significantly higher (10- to 20-fold) than Venus control cells (Figure 1H). This is consistent with PB analysis in the primary transplantation, showing no significant difference in the chimerism generated by Dppa5-overexpressing cells and fresh cells (Figure 1C). Thus, Dppa5 has a robust potential to improve reconstitution levels after transplantation, but it is not based on continuous self-renewing divisions as seen with HoxB4-transduced HSCs (Antonchuk et al., 2002).

Dppa5 Regulates ER Stress

Dppa5 is an RNA binding protein (Tanaka et al., 2006), but its biological role remains unknown. To clarify possible mechanisms and downstream effects of Dppa5, we performed proteomic analysis using 2D difference gel electrophoresis (2D DIGE) (Figure 2A). Differentially expressed protein spots detected in Dppa5-overexpressing cells were selected, and 11 upregulated and 17 downregulated proteins were identified (Figures 2B and 2C; Table S1). Upregulated proteins included a mitochondrial metabolism-related protein (Clpp), a redox-related protein (Peroxiredoxin 2 [Prdx2]), and amino acid/protein-processing proteins (Prosc, Ppp1cb, Eif1ax, etc.), suggesting that cell division and protein production are affected by Dppa5 (Figure 2C; Table S1). Consistent with these findings, we demonstrated that Dppa5-overexpressing HSCs in the BM of engrafted mice exhibit a significantly lower proportion of Ki67-negative cells than controls, indicating that Dppa5 enhances cell-cycle

Figure 1. Dppa5 Overexpression Profoundly Enhances the Long-Term Reconstitution Capacity of HSCs

(A) Schematic representation of lentiviral vectors used to overexpress Dppa5. The vector has the Venus-Dppa5 fusion protein expressed by the internal SFFV promoter, and the fusion protein is subsequently cleaved into the Venus and Dppa5 proteins by including a 2A sequence in the fusion protein. Empty vector (Venus alone) is used as the control vector.

(B) Experimental design of the reconstitution assay for Dppa5-overexpressing HSCs. A total of 50 $CD34^{-}48^{-}$KSL cells were sorted, transduced with Dppa5 or control lentiviral vectors, and cultured in serum-free medium supplemented with 100 ng/ml of mSCF and hTPO. Whole cultures were transplanted into lethally irradiated mice at days 2 and 14 in a competitive setting.

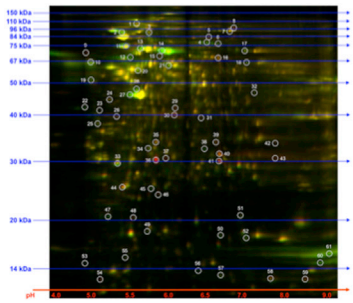
(C and D) Competitive reconstitution assay (primary transplantation). Values show percentages of donor-derived cells based on the congenic Ly-5.1/Ly-5.2 system (C) and $Venus^{+}$ cells (D) in the total engraftment. In (C), fresh cells that have not been cultured or transduced are used as control (mean ± SD; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; n = 10).

(E) Lineage distribution in $Venus^{+}$ cells of primary recipients at 16 weeks. For the fresh cell, sample data of $Venus^{-}$ cells are shown. Each bar represents the mean value (n = 10).

(F) Frequency of $Venus^{+}$ cells in $CD34^{-}$KSL, KSL, and Lin^{-} populations of BM from primary recipient mice at 16 weeks after transplantation (mean ± SD; $^{**}p < 0.01$; n = 6).

(G) Competitive reconstitution assay (secondary transplantation). Values show percentages of $Venus^{+}$ cells in the total engraftment (mean ± SD; $^{***}p < 0.001$; n = 10).

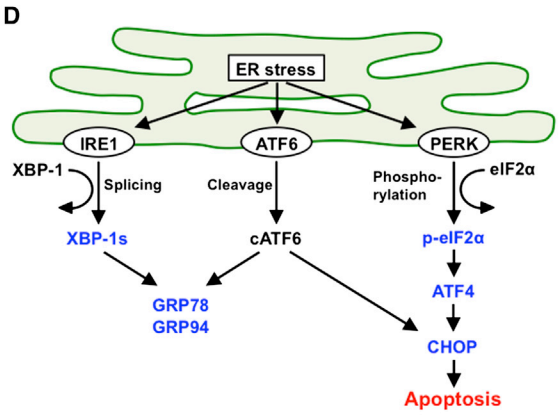
(H) Limiting dilution analysis for Dppa5-overexpressing HSCs. A total of 300 (experiment [Exp.] 1) or 200 (Exp.2 and Exp.3) $CD34^{-}48^{-}$KSL cells were transduced with control or Dppa5-overexpressing lentiviral vector and cultured for 14 days. Different numbers of cells were transplanted into lethally irradiated mice in a competitive setting. Positive mice were determined as showing more than 1% $Venus^{+}$ cells in total engraftment. HSC frequency was calculated using L-Calc software (STEMCELL Technologies).

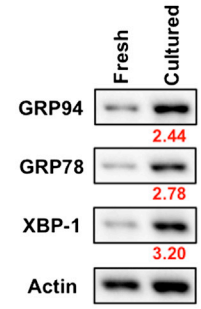


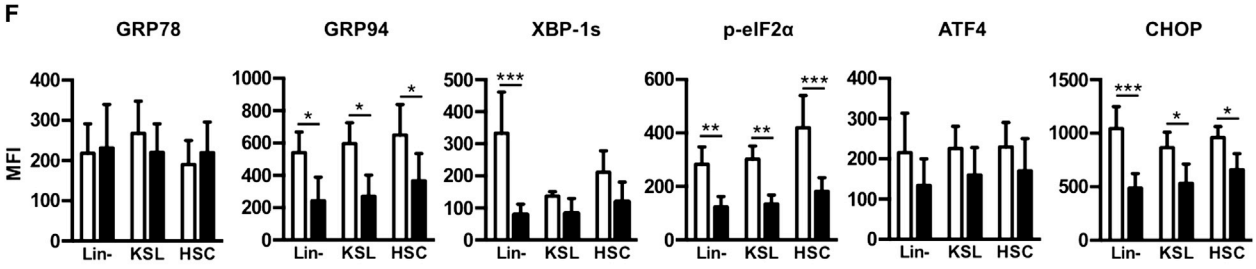
C

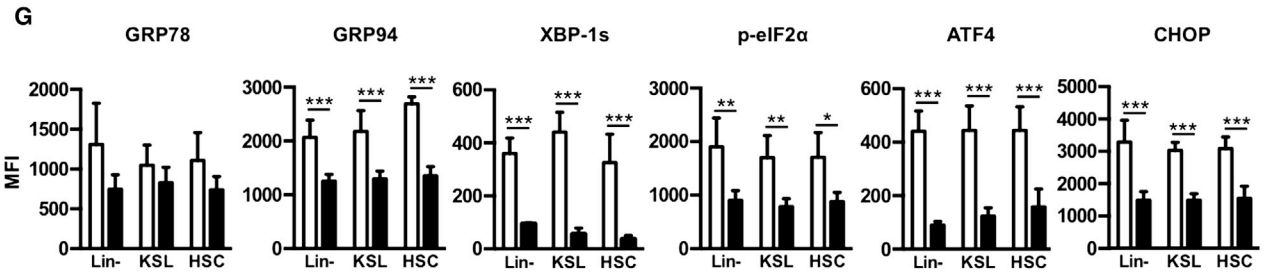
Proteins *up*-regulated by Dppa5
Mitochondrial metabolism
Clpp
Redox
Prdx2
Amino acid/protein processing
Prosc, Ppp1cb, Mthfd1, Eif1ax, Cct6a

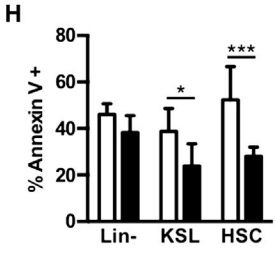
Proteins *down*-regulated by Dppa5
ER stress chaperones
GRP94, Txndc5, Pdia3, Calr, GRP78
Glycolytic enzymes
Pkm2, Pgk1
Calcium binding
Calr, S100a9
Cytoskeleton
Actg1, Tuba1b, Cotl1











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progression (Figure S1A). Downregulated proteins included ER stress chaperones (hsp90b1/GRP94, hspa5/GRP78, Txndc5, Pdia3, and Calreticulin), glycolytic enzymes (pyruvate kinase M2 [Pkm2] and Pgk1), calcium binding proteins (Calreticulin and S100a9), and cytoskeletal proteins (Actg1, Tuba1b, and Cotl1) (Figure 2C; Table S1). Interestingly, we have previously detected these same glycolytic enzymes as upregulated after HSC stimulation by Cripto, indicating enhanced glycolytic metabolism (Miharada et al., 2011). Pkm2 is a key enzyme in normal and tumor-specific glycolytic metabolism (the Warburg effect), with high expression in cancer cells (Warburg, 1956; Christofk et al., 2008). We analyzed glycolytic activity by measuring lactate production, and the results showed that Dppa5-overexpressing cells produced less lactate than control cells (Figure S1B). The metabolic change was also detected in the measurement of mitochondrial membrane potential using MitoTracker (MT) staining, showing higher MT intensity in immature cell populations of Dppa5-overexpressing cells (Figure S1C). The findings suggest that the metabolic activity in Dppa5-overexpressing cells was shifted from glycolysis to more oxidative phosphorylation.

GRP78 is a member of the hsp70 family and is normally expressed on ER membranes. GRP78 is a master regulator for the UPR as both a sensor of mis-/unfolded proteins and a chaperone helping with the folding. GRP94 is a member of the hsp90 family, which closely interacts with GRP78 and is implicated in many tumors (Luo et al., 2011; Dejeans et al., 2012; Luo and Lee, 2013). Pdia3 and Txndc5 are members of the protein disulfide isomerase (PDI) family that governs chaperone-mediated quality control of proteins by catalyzing disulfide bond formation (Benham, 2012). Reduced expression of these proteins indicates a lower level of ER stress in Dppa5-overexpressing cells.

The reaction of PDI produces ROS as a by-product (Zhang and Kaufman, 2008) that severely impairs the long-term reconstitution capacity of HSCs (Ito et al., 2006; Baudet et al., 2012). In addition, we also identified an increase of the Prdx2 protein that is known for its role in decreasing ROS levels (Agrawal-Singh et al., 2012; Figure 2C; Table S1). When the ROS levels were measured in Dppa5-overexpressing HSCs, a significant reduction in cytosolic ROS was observed compared with control (Figure S1D). These findings indicate that Dppa5 overexpression enhances protein synthesis and oxidative phosphorylation and simultaneously protects HSCs from ROS production and ER stress. These metabolic changes probably increase HSC function and activity and improve engraftment by Dppa5-overexpressing cells, even though HSC frequency is not increased.

Because Dppa5 is an RNA binding protein (Tanaka et al., 2006), we performed UV-crosslinking immunoprecipitation (UV-CLIP) experiments to identify direct RNA targets in hematopoietic cells. We detected *GRP94* and *Pkm2* as targets of the RNA binding function of Dppa5 as well as previously reported target transcripts, e.g., *Mapk6* and *Cdc42* (Figure S1E).

Dppa5 Protects HSCs from Apoptotic Signals Triggered by ER Stress Pathways

ER stress is mainly induced by the accumulation of un-/mis-folded proteins that exceed the capacity of the ER to fold or degrade them, triggering stress-response reactions (Luo and Lee, 2013). ER stress chaperones are critical components of the UPR, and many hsps have important roles in ER stress responses (Luo and Lee, 2013). Several studies have reported that they are involved in HSC regulation (Miharada et al., 2011; Zou et al., 2011; Wey et al., 2012). Based on the degree of ER stress, three distinct ER stress-response pathways are activated (Figure 2D). IRE1 and ATF6 mediate signals mainly enhancing protein folding and degradation of un-/mis-folded proteins (survival signals/early ER stress response), but severe and continuous stress triggers cell-cycle arrest and apoptosis mainly governed by protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) (apoptosis signals/late ER stress response) (Lin et al., 2007; Walter and Ron, 2011). Ex vivo culture enforces higher proliferation and protein synthesis in a suboptimal environment that may lead to a significant augmentation of un-/mis-folded proteins. In addition, albumin, an indispensable component of serum-free media, is known to induce ER stress and subsequent apoptosis signals (Ohse et al., 2006). We therefore hypothesized that in vitro culture triggers ER stress-response pathways in hematopoietic stem/progenitor cells. We measured accumulation of proteins encoded by ER stress-response genes. Protein levels of GRP78, GRP94, and XBP-1 were clearly increased (Figure 2E), suggesting that even after

Figure 2. Dppa5 Mediates Its Effects in HSCs through Regulation of ER Stress

(A) Experimental design of the proteomic assay. $CD34^{-}48^{-}$KSL cells were transduced with control or Dppa5-overexpressing vector, cultured for 2 days, and transplanted to lethally irradiated mice. Four months later, $Venus^{+}/Lin^{-}/c\text{-}kit^{+}$ cells were isolated from the BM. For the mock control, $Venus^{-}/Lin^{-}/c\text{-}kit^{+}$ cells were isolated from both control and Dppa5 recipients. Cells were lysed and analyzed with the 2D DIGE analysis.

(B) Merged image of the 2D DIGE analysis showing up- and downregulated proteins. Differentially expressed spots were selected, indicated by white circles. Green indicates protein from Venus control cells; red designates protein from Dppa5-overexpressing cells.

(C) Summary of identified proteins. Proteins that are up-/downregulated in the 1.5- to 3.5-fold range are listed. For more detailed information, see Table S1.

(D) Schematic overview of ER stress-response branches mediated through IRE1, ATF6, and PERK. Genes highlighted in blue are analyzed in the following studies: XBP-1s, spliced XBP-1; cATF6, cleaved ATF6; and p-eIF2α, phosphorylated eIF2α.

(E) Natural induction of ER stress by in vitro culture. Western blot analysis shows increased ER stress chaperones in hematopoietic stem/progenitor cells. $c\text{-}kit^{+}$ cells from BM that were freshly isolated or cultured for 3 days were harvested, and protein expression of GRP94, GRP78, and XBP-1 was measured. Each value shows fold change compared to Fresh sample after normalization with Actin.

(F and G) Intracellular staining of proteins encoded by ER stress-response genes demonstrates reduced ER stress in HSCs and progenitors. $c\text{-}kit^{+}$ cells were transduced with control or Dppa5 lentiviral vector followed by fluorescence-activated cell sorting (FACS) analysis of HSCs and progenitors. Mean fluorescence intensity (MFI) of the proteins in Lin^{-}, KSL, and $CD48^{-}$KSL (HSC) cells at day 3 (F) and day 6 (G) is shown. White bar indicates control; black bar designates Dppa5 (mean ± SD; $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$; n = 4).

(H) Annexin V staining in Dppa5-overexpressing cells. Transduced and cultured $c\text{-}kit^{+}$ cells were analyzed at day 6. White bar indicates control; black bar designates Dppa5 (mean ± SD; $^{***}p < 0.001$; $^{*}p < 0.05$; n = 6).

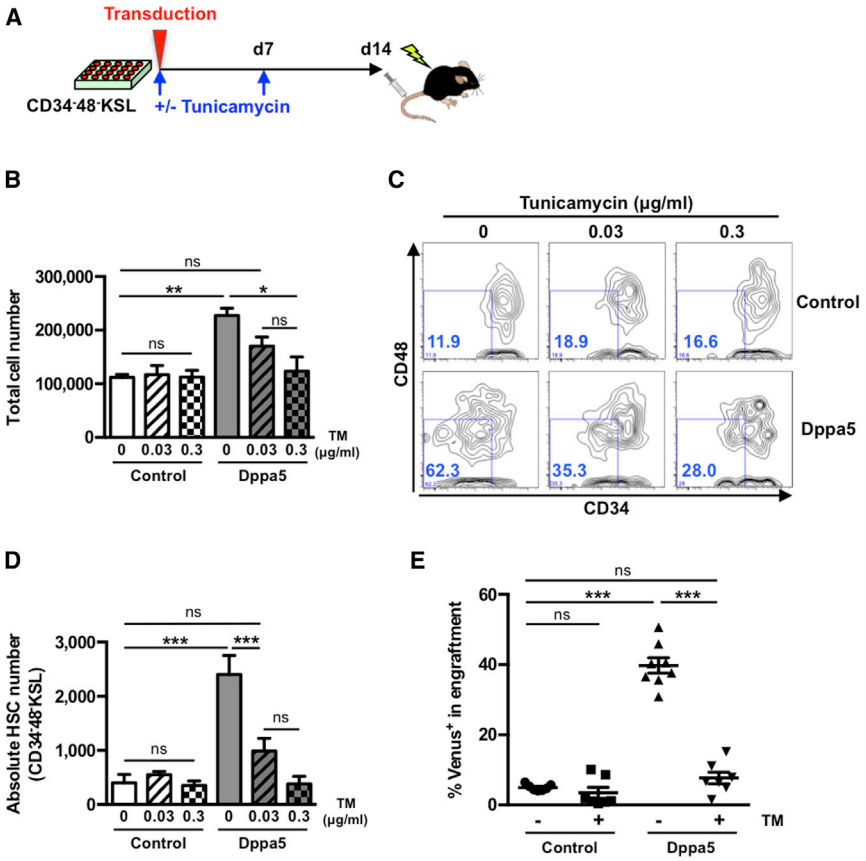


Figure 3. Induction of ER Stress Constrains Dppa5 Function

(A) Experimental design of the reconstitution assay for Dppa5-overexpressing HSCs cultured with TM. A total of 50 $CD34^{-}48^{-}$KSL cells were sorted, transduced with Dppa5 or control lentiviral vectors, and cultured with or without TM. Whole cultures were transplanted into lethally irradiated mice at day 14 in a competitive setting.

(B) Cell count for control or Dppa5-overexpressing KSL cells cultured with TM for 14 days (mean ± SD; *$p < 0.05$; **$p < 0.01$; ns, not significant; $n = 4$).

(C) Representative FACS pattern of the $CD34^{-}CD48^{-}$ population in control or Dppa5-overexpressing cells treated with TM for 14 days. Percentage of gated cells represents the $Venus^{+}$ KSL population.

(D) Calculation of the absolute number of phenotypic HSCs for control or Dppa5-overexpressing cells after TM treatment starting from 50 cells (mean ± SD; ***$p < 0.001$; $n = 4$).

(E) Competitive reconstitution analysis of control and Dppa5-overexpressing cells treated with 0.3 μg/ml of TM for 14 days. Percentages of $Venus^{+}$ cells in total engraftment are shown (***$p < 0.001$; $n = 10$).

a short cultivation period, ER stress-response pathways are activated. Interestingly, several hsps have distinct expression patterns in different hematopoietic cell populations, especially in the LT-HSC population, e.g., Pdia1 shows lower expression in $CD34^{-}$KSL cells. In contrast, Dnajc1 and Dnajc4 show higher levels (Figure S1F), suggesting that susceptibility to ER stress in HSCs is different than that in other populations of hematopoietic cells.

To further study the involvement of ER stress response connected to Dppa5 function in HSCs, we performed intracellular staining of ER stress proteins in Dppa5-overexpressing cells. BM-derived c-kit^{+} cells were transduced with control or Dppa5 lentiviral vector and cultured in vitro. In Dppa5-overexpressing cells, protein levels of ER stress-response genes, particularly late signaling molecules (e.g., phosphorylated eIF2α, and ATF4), were significantly lowered (Figures 2F and 2G). In addition, expression of CCAAT-enhancer-binding protein (C/EBP) homologous protein (CHOP), which triggers ER stress-induced apoptosis, was significantly decreased. When we measured frequency of apoptotic cells using annexin V staining, the Dppa5-overexpressing HSC population contained significantly fewer apoptotic cells (Figure 2H). We also saw a decrease in the apoptosis of c-kit^{+}/Sca-I^{+}/$Lineage^{-}$ (KSL) cells, but no difference in lineage-negative (Lin^{-}) cells. These findings suggest that Dppa5 specifically protects primitive populations from culture-induced apoptosis by reducing ER stress levels.

Chemically Induced ER Stress Counteracts Dppa5 Function

To further confirm that reduction of ER stress level is the key function of Dppa5, we asked whether induction of severe ER stress inhibits the effects of Dppa5 on HSC function. Tunicamycin (TM) is a mixture of homologous antibiotics and is known to induce UPR leading to severe ER stress (Wang et al., 2011). Dppa5-overexpressing $CD34^{-}$ 48^{-}KSL cells and control cells were cultured with or without TM for 14 days (Figure 3A). Lower concentrations did not show reduction in the cell growth of control cells (Figure 3B), whereas a higher concentration (3 μg/ml) showed a clear and immediate increase in CHOP expression and reduced viability, indicating toxicity of TM using that concentration (Figure S2; data not shown). Cell growth enhanced by Dppa5 was effectively inhibited by TM treatment at the lower (0.03 and 0.3 μg/ml), nontoxic, concentrations (Figure 3B). Furthermore, no significant difference was seen in the frequency of the $CD34^{-}48^{-}$KSL population between control cells and Dppa5-overexpressing cells treated with TM at the 0.03 and 0.3 μg/ml concentrations (Figure 3C). When we calculated the absolute number of $CD34^{-}48^{-}$KSL cells, it was clear that the advantage of Dppa5 overexpression was diminished by TM treatment in a dose-dependent manner (Figure 3D). We transplanted Dppa5-overexpressing cells treated with TM (0.3 μg/ml) for 14 days in vitro into lethally irradiated mice, and the findings showed no difference in chimerism compared to control (Figure 3E). Taken together, these findings show that the induction of severe ER stress counteracts the increase in engraftment and in the absolute number of $CD34^{-}48^{-}$KSL cells generated by Dppa5.

Reduced Dppa5 Expression Profoundly Impairs Long-Term Reconstitution of HSCs due to Increased ER Stress

In order to ask whether Dppa5 regulates ER stress in HSCs and progenitors in vivo, we analyzed the expression level of Dppa5 in different hematopoietic cell populations. We tested HSCs that are positive or negative for cell surface GRP78 because cell surface GRP78 distinguishes dormant, myeloid-biased HSCs (GRP78$^+$HSC) and active, lymphoid-biased HSCs (GRP78$^-$HSC) (Miharada et al., 2011). *Dppa5* mRNA was expressed at significantly higher levels in the GRP78$^-$HSCs compared to mature hematopoietic populations (Figure 4A). We also found a trend toward higher expression of *Dppa5* in GRP78$^-$HSCs compared to the GRP78$^+$HSCs. These findings prompted us to ask if reduction of Dppa5 has an impact on the long-term reconstitution capacity of HSCs. We performed knockdown experiments with Dppa5-specific small hairpin RNAs (shRNAs) inserted into lentiviral vectors that express Venus as a reporter gene (Figure 4B). KSL cells were sorted and transduced with shRNA lentivirus, followed by competitive transplantation analysis (Figure 4C). One month after transplantation, Venus$^+$ CD150$^+$KSL cells were isolated from recipient mice, transcription levels of *Dppa5* were measured by quantitative real-time PCR, and the results showed that three out of four shRNA sequences (shRNA [sh]-#2, sh-#3, and sh-#4, Figure 4D) clearly reduced Dppa5 expression compared to the scrambled (Scr) control vector. The same three shRNA sequences significantly reduced engraftment of transduced hematopoietic cells over time (1–4 months), with no significant difference observed in lineage distribution (Figures 4E and S3A). The lower engraftment by the shRNAs was also seen in the BM of recipient mice (Figure S3B). These results suggest that endogenous Dppa5 expression is critical for the long-term reconstitution capacity of HSCs.

To ask if the impaired HSC function is due to increased apoptosis triggered by the ER stress-response pathway, we analyzed ER stress levels in the engrafted HSCs using intracellular staining. One month after transplantation, BM cells derived from the engrafted mice were harvested, and protein levels of ER stress-response genes in the Venus$^+$ donor-derived cells were measured. Flow cytometry analyses observed significantly higher levels of XBP-1s, p-eIF2α, ATF4, and CHOP in sh-#3-expressing HSCs (Figure 4F). In addition, sh-#2 and sh-#4 showed higher levels of XBP-1s and CHOP (Figure 4F). The induction of late ER stress pathway in sh-#3 and sh-#4 was consistent with an increased number of apoptotic cells (Figure 4G). Of note, higher ER stress levels in the sh-#2- and sh-#4-expressing cells were detected only in the HSC population, whereas sh-#3 showed the elevated ER stress level also in other populations, indicating that reconstitution levels generated by the donor HSCs are highly correlated to the ER stress level and apoptosis (Figures 4E–4G). Taken together, Dppa5 expression is critical for HSC function because reduction by shRNA leads to elevated ER stress and apoptosis.

ER Stress Reduction Highly Improves HSC Function and Reconstitution

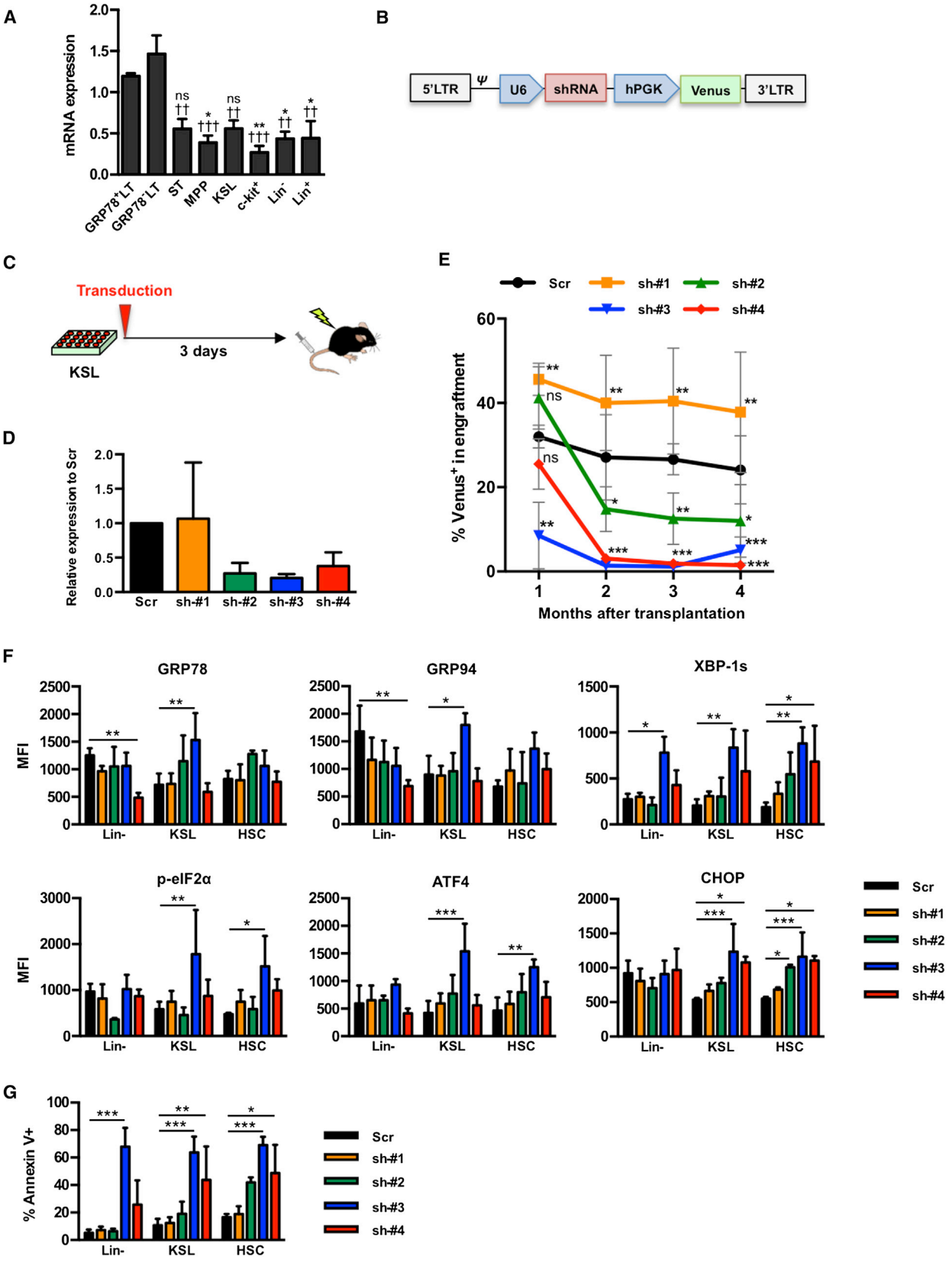
Because Dppa5 significantly improves HSC function by diminishing ER stress levels, we hypothesized that chemical treatment reducing ER stress might mimic the effects of Dppa5. Tauroursodeoxycholic acid (TUDCA) is a bile acid that is known to enhance protein folding, resulting in reduced ER stress (Özcan et al., 2006; Ozcan et al., 2009; Berger and Haller, 2011). Using TUDCA treatment, we could show that in vitro culture of c-kit$^+$ cells showed dose-dependent reduction in the expression levels of the UPR genes (Figure S4A). To confirm the ER stress reduction by TUDCA treatment in HSCs, we measured protein levels of ER stress-response genes in cultured cells. BM-derived c-kit$^+$ cells were cultured with or without 60 μM of TUDCA, and intracellular staining was performed. Expression levels of ER stress-response genes, including XBP-1s and CHOP, were significantly lower in TUDCA-treated HSCs (Figures 5A, 5B, and S4B). After 6 days in culture, apoptosis was preferentially induced in HSCs, but TUDCA treatment inhibited apoptosis induction (Figure 5C).

Recent findings indicate that ER stress can affect cellular metabolism (Özcan et al., 2006; Fang et al., 2010; Wang et al., 2011). We asked whether metabolic changes were seen in hematopoietic stem/progenitor cells following changes in ER stress. In contrast to fresh cells, cultured c-kit$^+$ cells show a higher Pkm2 protein level along with an increase in GRP78 and GRP94, but TUDCA-treated cells show a clear decrease in Pkm2, GRP78, GRP94, and XBP-1 (Figure S4A). The 60 μM TUDCA-treated cells showed a dose-dependent decrease in MT, ROS intensity, and lactate production (Figures S4C–S4E; data not shown). These findings suggest that ER stress levels may also affect the metabolic condition of HSCs.

To assess how the decreased ER stress affects HSC potential, we cultured CD34$^-$48$^-$KSL cells with TUDCA for 14 days (Figure 5D). Surprisingly, TUDCA addition increased total hematopoietic cell number (Figure 5E) and the frequency of CD34$^-$48$^-$KSL cells (Figure 5F). The absolute number of CD34$^-$48$^-$KSL cells was also increased, although the highest concentration (60 μM) did not have additional effects (Figure 5G). Finally, we transplanted the TUDCA-treated cells into lethally irradiated mice. Cells cultured with 60 μM TUDCA for 14 days showed a robust increase in reconstitution levels compared to control cells, whereas 6 μM TUDCA showed only a modest insignificant increase in engraftment (Figure 5H). Thus, chemical treatment reducing ER stress is a robust method to maintain the long-term reconstitution capacity of HSCs ex vivo.

DISCUSSION

Here, we report that Dppa5 is a critical regulator for HSC function because overexpression leads to a robust increase in reconstitution capacity (Figures 1D and 1G), whereas knockdown of Dppa5 significantly reduces long-term reconstitution (Figure 4E). Proteomic and flow cytometry analyses revealed that Dppa5 expression is closely connected to ER stress. Dppa5 overexpression decreased expression levels of ER stress-response genes and ratio of apoptotic cells, whereas knockdown of Dppa5 resulted in increased ER stress and apoptosis (Figures 3C, 3D, and 5F). In support of our findings, the involvement of ER stress in HSC regulation was recently reported in a hypoxia-inducible factor-2α knockdown model of human HSCs (Rouault-Pierre et al., 2013). We show that HSCs are more susceptible to ER stress because the increase of ER



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stress-response genes and subsequent activation of apoptosis were preferentially detected in the HSC population (Figures 2F–2H, 4F, and 4G). hsps have been implicated as important regulators of HSCs (Miharada et al., 2011; Zou et al., 2011; Wey et al., 2012), and the apparent vulnerability to ER stress that we report here may partly be explained by distinct expression levels of hsps in HSCs (Figure S1F). Our findings and recent studies suggest that ER stress may be important in the physiological regulation of tissue homeostasis and regeneration and in the pathogenesis of malignant and nonmalignant disorders (Özcan et al., 2006; Wang and Kaufman, 2012; Rouault-Pierre et al., 2013).

Interestingly, the improved reconstitution capacity of Dppa5-overexpressing cells is independent from an increase in HSC number (Figure 1H). In the proteomic analysis from long-term-reconstituted mice, we detected altered protein profiles indicating reduced ER stress in Dppa5-overexpressing cells. Although the primary recipient mice engrafted with 2-day cultured Dppa5-overexpressing HSCs showed no difference in PB and BM (Figures 1C and 1F), the cells showed significantly higher reconstitution level in the secondary transplantation compared to control cells (Figure 1G). This suggests that quality of HSCs, e.g., ER stress levels, may be equally important for reconstitution capacity as the number of phenotypic HSCs. Recent studies have also demonstrated that the metabolic condition of HSCs is an important determinant of their activity, preservation, and survival. HSC metabolism is highly dependent on glycolysis in the quiescent state, and that reduced glycolytic metabolism in HSCs can lead to a loss of quiescence and even stem cell exhaustion (Gan et al., 2010; Gurumurthy et al., 2010; Nakada et al., 2010; Takubo et al., 2010, 2013). Recently, we have shown that enhanced glycolytic activity can maintain functional HSCs in culture by mimicking their metabolic properties in vivo (Miharada et al., 2011; Takubo et al., 2013). In contrast, our current findings demonstrate that the elimination of ER stress during the ex vivo culture represents an innovative approach to treating HSCs and proposes that HSC potential can be preserved or enhanced by reducing stress signals.

The findings presented here have important implications for functional preservation of HSCs ex vivo. A high fraction of HSCs are quiescent in vivo, and in order to increase their number, particularly in vitro, cell growth has to be enhanced to achieve expansion. However, this enforces HSCs to undertake strenuous proliferation that induces ER stress and subsequent ROS accumulation. Importantly, reduction of ER stress by TUDCA treatment in vitro resulted in a robust increase in engraftment of functional HSCs (Figure 5E). The use of chemical chaperones, like TUDCA, may therefore become instrumental in developing new methods to expand/maintain functional HSCs ex vivo and may have a potential to improve clinical transplantation of HSCs in the future.

In conclusion, this work depicts Dppa5 as an important regulator of survival and functional HSC activation through its regulation of ER stress. Reduction of ER stress by overexpression of Dppa5 or a chemical chaperone reduced apoptosis and improved functional reconstitution of HSCs (Figure 5H). In contrast, downregulation of Dppa5 by shRNAs in vivo profoundly impaired the long-term reconstitution ability of HSCs due to elevated ER stress levels, suggesting that ER stress regulation is physiologically important for proper HSC function in vivo. Our findings highlight the need to minimize ER stress in vitro for the development of stem cell expansion protocols to avoid compromising the functionality of HSCs. Therefore, regulation of protein quality control and ER stress regulation may be important to achieve HSC activation and improved BM reconstitution.

EXPERIMENTAL PROCEDURES

All animal experiments were approved by the Lund University Animal Ethical Committee.

Lentiviral-Mediated Overexpression

A total of 50–100 $CD34^-CD48^-$KSL cells were purified from B6SJL (Ly-5.1) mice and transduced with Dppa5 or control lentiviral vector. Cells were cultured in serum-free media (StemSpan serum-free expansion medium [SFEM]; STEMCELL Technologies) supplemented with 100 ng/ml of mouse stem cell factor (mSCF) and human thrombopoietin (hTPO; PeproTech) for 2 weeks.

Competitive reconstitution analysis was performed by transplanting cultured cells into lethally irradiated (900 cGy) C57BL/6 (Ly-5.2) mice along with 200,000 total BM competitor cells from Ly-5.1/Ly-5.2 mice. Every 4 weeks after transplantation, PB was collected from recipient mice and analyzed for chimerism and lineage distribution. BM of recipient mice was analyzed for chimerism and lineage distribution 16 weeks after transplantation. Five million

Figure 4. Reduced Expression of Dppa5 Triggers Elevated ER Stress and Severely Impairs the Long-Term Reconstitution Capacity of HSCs

(A) Quantitative real-time PCR analysis of *Dppa5* mRNA expression in $GRP78^+$LT ($GRP78^+CD34^-Flt3^-$KSL), $GRP78^-$LT ($GRP78^-CD34^-Flt3^-$KSL), ST ($CD34^+Flt3^-$KSL), MPP ($CD34^+Flt3^+$KSL), KSL, c-kit^+, Lin^-, and Lin^+ cells. Each value is normalized to hypoxanthine-guanine phosphoribosyltransferase expression, and mean ± SD is shown. Significance is shown when compared with $GRP78^+$ HSCs (*$p < 0.05$; **$p < 0.01$; n = 3) and $GRP78^-$ HSCs ($^{\dagger\dagger}p < 0.01$; $^{\dagger\dagger\dagger}p < 0.001$; n = 3).

(B) Schematic representation of lentiviral vectors used to express Dppa5-targeting shRNAs. Nontargeting RNA sequence (Scr) is used as the control vector.

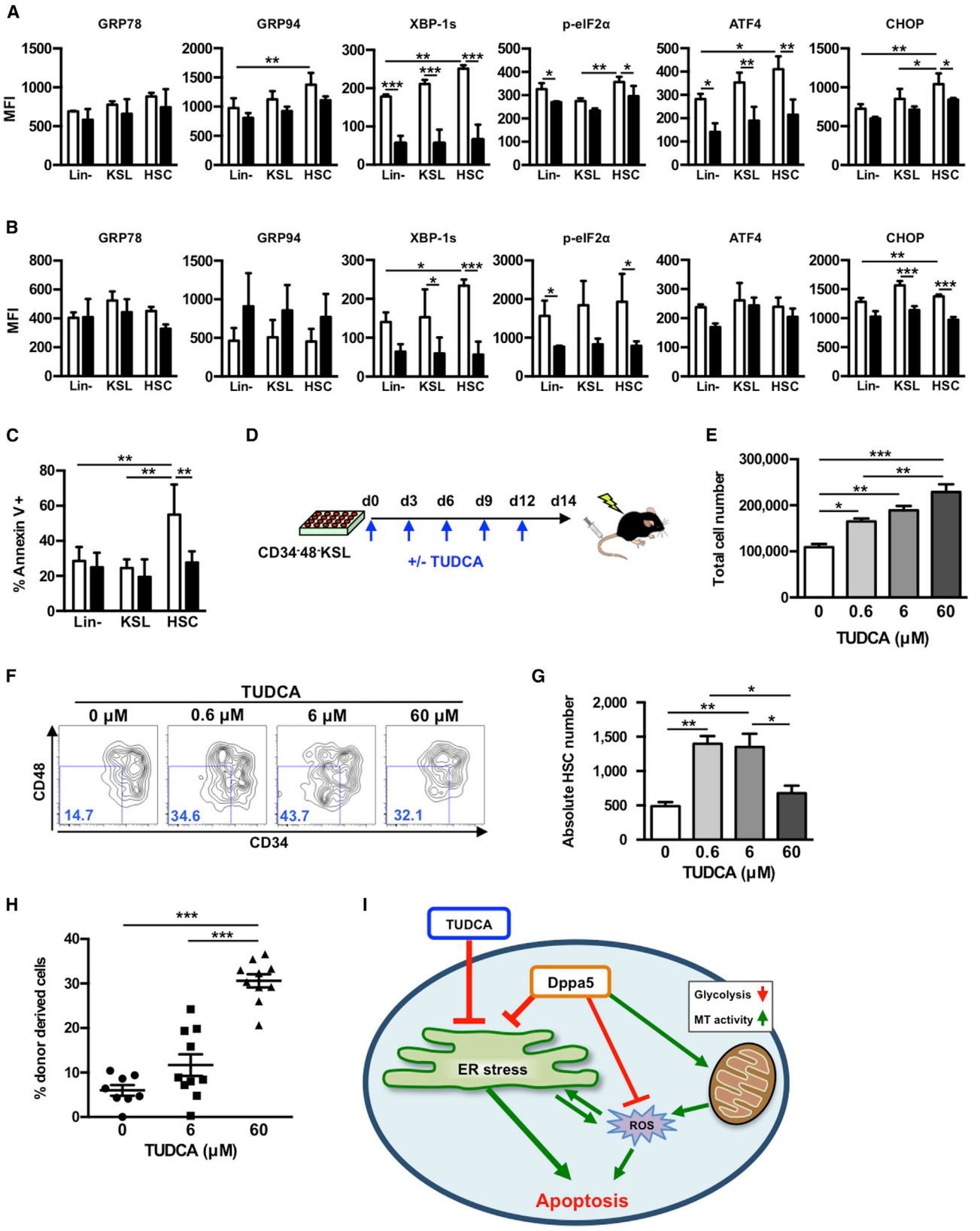
(C) Experimental design of the reconstitution assay to test HSCs expressing the various shRNAs. KSL cells were sorted and transduced with sh-#1, sh-#2, sh-#3, and sh-#4 and Scr control lentiviral vectors. After 3 days, the culture equivalent to 5,000 starting cells (per recipient) was transplanted into lethally irradiated mice in a competitive setting.

(D) Validation of Dppa5 knockdown efficiency in engrafted HSCs. $Venus^+$ $CD150^+$KSL cells were sorted from recipient mice, and mRNA expression levels of Dppa5 were determined by quantitative real-time PCR.

(E) Competitive reconstitution of Dppa5 knockdown cells. Percentages of $Venus^+$ cells in the total engraftment are shown (mean ± SD; n = 6). Significance compared to Scr: **$p < 0.01$; ***$p < 0.001$.

(F) Intracellular staining of proteins encoding ER stress-response genes. c-kit^+ cells were isolated from recipient mice engrafted for 1 month with HSCs expressing shRNA, and protein levels of ER stress-response genes in $Venus^+$ cells were measured. Mean fluorescence intensity (MFI) of the proteins in Lin^-, KSL, and $CD150^+$KSL (HSC) cells is shown (mean ± SD; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n = 3).

(G) Apoptosis assay (annexin V staining) for engrafted shRNA-expressing cells. Percentage of annexin V positive within Lin^-, KSL, and $CD150^+$KSL (HSC) cells is shown (mean ± SD; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; n = 3).



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BM cells from primary recipients were transplanted into lethally irradiated secondary recipient mice. PB from secondary recipients was analyzed using the same approach as with primary recipients.

shRNA-Mediated Knockdown Analysis

Dppa5-specific shRNA lentiviral vectors (MISSION shRNA) were obtained from Sigma-Aldrich. Scr sequence was used as a control. The puromycin-resistance gene was removed from the vector and replaced by Venus.

For competitive reconstitution analysis, KSL cells were purified from Ly-5.1 mice and transduced with Dppa5 shRNA lentiviral vectors. After culture for 3 days, the equivalent of 5,000 cells was transplanted into lethally irradiated Ly-5.2 recipient mice with competitor cells.

HSC Culture with ER Stress Inducer/Inhibitor

Sorted cells were cultured in SFEM supplemented with mSCF and hTPO, as described above for 14 days. TM (Sigma-Aldrich) was dissolved in 100% ethanol and used in the concentrations of 0.03, 0.3, and 3 μg/ml. TUDCA (Sigma-Aldrich) was dissolved in 100% ethanol and used in the concentrations of 0.6, 6, and 60 μM. Competitive reconstitution analysis was performed using the same approach described above.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.04.056.

AUTHOR CONTRIBUTIONS

K.M. designed and conducted the project. K.M. and V.S. performed the experiments. S.K. directed the research. All authors wrote the manuscript.

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Figure 5. Reduction of ER Stress by a Chemical Chaperone Improves HSC Function

(A and B) Intracellular staining of proteins encoding ER stress-response genes in TUDCA-treated cells. c-kit$^+$ cells were cultured with or without 60 μM of TUDCA. Mean fluorescence intensity (MFI) of the proteins in Lin$^-$, KSL, and CD48$^-$KSL (HSC) cells at day 3 (A) and day 6 (B) is shown. White bar indicates control; black bar designates TUDCA-treated cells (mean ± SD; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; $n = 4$).

(C) Apoptosis assay (annexin V staining) for TUDCA-treated cells. c-kit$^+$ cells cultured with or without 60 μM were analyzed at day 6. White bar indicates control; black bar designates TUDCA-treated cells (mean ± SD; **$p < 0.01$; $n = 6$).

(D) Experimental design of the reconstitution assay for HSCs cultured with TUDCA. A total of 100 CD34$^-$48$^-$KSL cells were sorted and cultured with or without TUDCA on the days indicated by arrows. Whole cultures were transplanted into lethally irradiated mice at day 14 in a competitive setting.

(E) Enumeration of total cell number produced from HSCs cultured with TUDCA (mean ± SD; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; $n = 4$).

(F) Representative FACS pattern of CD34$^-$48$^-$KSL cells cultured with TUDCA for 14 days.

(G) Enumeration of CD34$^-$48$^-$KSL cells cultured with TUDCA (mean ± SD; *$p < 0.05$; **$p < 0.01$; $n = 4$).

(H) Competitive reconstitution analysis of TUDCA-treated HSCs. A total of 100 CD34$^-$48$^-$KSL cells were sorted and cultured with TUDCA (0, 6, and 60 μM). After 14 days in culture, cells were transplanted to recipient mice. Percentages of donor-derived cells in total engraftment at the 12th week are shown (mean ± SD; ***$p < 0.001$; $n = 7$).

(I) A model is depicted for the regulation of HSC activity and reconstitution ability mediated by Dppa5 and TUDCA through reduction in ER stress.

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