Molecular regulation of human hematopoietic stem cells
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Chapter 4

Human hematopoietic stem cell integrity is guarded by the Unfolded Protein Response

Peter van Galen, Antonija Kreso, Nathan Mbong, Erno Wienholds, David G. Kent, Timothy Fitz-Maurice, Joseph E. Chambers, Stephanie Xie, Elisa Laurenti, Karin Hermans, Kolja Eppert, Stefan J. Marciniak, Jane C. Goodall, Anthony R. Green, Bradly G. Wouters and John E. Dick

Currently under review.
Three branches of the UPR are activated upon ER stress: IRE-1, PERK and ATF6. IRE-1 splices cytosolic XBP1 mRNA to enable translation of the XBP1s transcription factor, which upregulates chaperones and ER-associated degradation (ERAD) machinery to resolve ER stress. PERK initiates a different branch of the UPR through phosphorylation of eIF2α, which attenuates global protein synthesis, thus permitting time to restore ER homeostasis. Prolonged ER stress leads to PERK signaling-mediated upregulation of the proapoptotic transcription factor CHOP and its target GADD34. GADD34 dephosphorylates eIF2α leading to restoration of global protein translation. However, if ER stress is not resolved, GADD34 upregulation can lead to further accumulation of misfolded proteins, oxidative stress and apoptosis. Yellow highlighted arrows indicate transcriptional regulation. P: phosphorylation, ERAD: ER-associated degradation.
Chapter 4. The unfolded protein response governs integrity of the human hematopoietic stem cell pool during stress

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4.1 Introduction

The blood system is sustained by a pool of hematopoietic stem cells (HSC) that are long-lived due to their capacity for self-renewal. A consequence of longevity is exposure to stress stimuli including reactive oxygen species (ROS), nutrient fluctuation, and DNA damage (Geiger et al., 2013; Yahata et al., 2011). Damage that occurs within stressed HSC must be tightly controlled to prevent either loss of function or the clonal persistence of oncogenic mutations that increase the risk of leukemogenesis (Jan et al., 2012; Rossi et al., 2008). Despite the importance of maintaining cell integrity throughout life, how the HSC pool achieves this and how individual HSC respond to stress remains poorly understood. Many sources of stress cause misfolded protein accumulation in the endoplasmic reticulum (ER) and subsequent activation of the unfolded protein response (UPR) that enables the cell to either resolve the stress or initiate apoptosis (Rutkowski and Kaufman, 2004; Walter and Ron, 2011). Here we show that human HSC are predisposed to UPR mediated apoptosis compared to closely related early progenitors (EP). Following ER stress in HSCs, the PERK branch of the UPR is strongly activated, causing ATF4, CHOP and GADD34 upregulation leading to apoptosis. In contrast, EP exhibit an adaptive response to stress leading to their survival. Modulation of UPR signaling in HSC by overexpression of the co-chaperone ERDJ4 increases HSC repopulation capacity in xenograft assays, directly linking the UPR to HSC function. Since the UPR is a focal point where different sources of stress converge, our study provides a framework for understanding how stress signaling is coordinated within the hematopoietic hierarchy and integrated with stemness. Moreover, our results may open up an avenue to exploit UPR signaling as a means to improve clinical transplantation of HSC. More broadly, these findings reveal how various sources of stress lead to clearance of individual HSC to prevent propagation of damaged HSC providing insight into how the stem cell pool maintains clonal integrity.
4.2 Results and discussion

The human hematopoietic hierarchy has recently been delineated at the single cell level, enabling precise isolation of HSC and progenitor cells (HSPC) (Doullatov et al., 2010; Majeti et al., 2007; Notta et al., 2011) (Sup. Table 1). We performed pathway analysis using the gene expression signature of highly purified HSC from lineage depleted cord blood (CB) (Laurenti et al., 2013), and identified components of the UPR to be enriched in HSC compared to progenitor cells (Fig. 1a-b). The UPR encompasses the IRE-1, PERK and ATF6 pathways (Walter and Ron, 2011) (Sup. Fig. 1a). Quantitative PCR showed that several genes of the PERK signaling branch were more highly expressed in CD34⁺CD38⁻ (HSPC) compared to CD34⁺CD38⁺ (EP) fractions: PERK (1.8-fold), ATF4 (1.5-fold) and its transcriptional targets XBP1 (2.3-fold), ERDJ4 (2.1-fold) and CHOP (1.7-fold), and the CHOP targets GADD34 (1.3-fold) and ERO1LB (2.1-fold, Fig. 1c, Sup. Fig. 1b-e, Sup. Table 2). HSPC-enriched expression of CHOP and ERDJ4 was also seen in adult bone marrow (BM, Sup. Fig. 1f). As well, expression of ATF6 and the ER resident chaperone GRP94 was enriched in CB HSPC compared to EP (Fig. 1d-e). However, splicing of XBP1 mRNA, which is representative of IRE1 activity, was lower in HSPC compared to EP (2.8-fold, Fig. 1f). Taken together, gene expression analysis of HSPC and EP fractions suggests differential activation of UPR branches, with increased expression of PERK dependent genes and decreased activity of IRE-1 in HSC.

To examine whether differential basal UPR gene expression reflects distinct ER stress responses in HSC and EP, we used two chemical inducers of ER stress, Thapsigargin (Tg) and Tunicamycin (Tm). Tg disrupts Ca²⁺ homeostasis by inhibiting SERCA in the ER membrane, depleting Ca²⁺ from the ER and rapidly activating all three branches of the UPR (DuRose et al., 2006). Treatment of sorted HSPC and EP with Tg resulted in upregulation of the canonical UPR target genes GRP94, GRP78 and ERDJ4 (Fig. 1g, Sup. Fig. 2a). Interestingly, after 30 minutes, the ratio of spliced over total XBP1 was increased by 9.0±1.1 fold in EP but only 2.4±0.3 fold in HSPC cells (Fig. 1g). The level of XBP1 splicing was diminished after 6 hours, consistent with quick attenuation of IRE-1 activity (Lin et al., 2007). This indicates that differential XBP1 splicing between HSC and EP under steady state conditions is exaggerated upon Tg-induced ER stress, consistent with repressed IRE-1 pathway activity in HSC.

Tm blocks synthesis of N-linked glycoproteins, causing accumulation of unfolded proteins in the ER (DuRose et al., 2006). Tm treatment resulted in higher upregulation of the canonical UPR genes GRP94, GRP78 and ERDJ4 in HSPC compared to EP (Fig. 1h, Sup. Fig. 2b). The level of XBP1 splicing by the IRE1 branch of the UPR was not different between CB-derived HSPC and EP following Tm treatment. In contrast, the PERK pathway target CHOP was more upregulated in HSPC compared to EP following addition of 0.6 µg/mL Tm (HSPC: 21±1.8-fold, EP: 5.9±0.4-fold, Fig. 1h). As well,
Figure 1 | Elevated expression of PERK branch genes of the UPR in HSC compared to EP and further amplification following Tm-induced stress. a, b, Enrichment of UPR-related genes in human HSC compared to progenitors. (a) CD49f+ HSC-enriched genes were analyzed for GO category overrepresentation. Node size represents the number of genes corresponding to that node. White, yellow and orange node color correspond to FDR<0.15, <0.1 and <0.01, respectively. (b) Heatmap showing 40 UPR-related genes, derived from nodes in (a), with differential expression between HSC and progenitors by expression array (FDR<0.05). c-f, Expression of key UPR genes in sorted HSPC and EP fractions. mRNA levels of (e) PERK pathway components, (d) ATF6, (e) GRP78 and GRP94 and (f) IRE1 and spliced XBP1 were measured by qPCR. Solid arrows indicate some transcriptional relationships. Expression was normalized to the average of GAPDH, ACTB and PBGD. Results are shown as mean±SEM of n=6 CB. g, h, UPR branch activation depends on cell type and stressor. CB HSPC and EP fractions were sorted and plated in the presence of (g) 0.2 µM Tg or (h) 0.6 µg/mL Tm. mRNA was isolated after 0.5, 1, 6, 16 and 40 hours to measure mRNA levels of GRP94, spliced and total XBP1, and CHOP by qPCR. DMSO treated controls were the same between (g) and (h). Expression was normalized to GAPDH. Data is shown as mean±SEM of n=3 CB, p-value was calculated based on treated/control cells and indicates differential response between HSPC and EP. HSC: hematopoietic stem cell, MPP: multipotent progenitor, MLP: multilymphoid progenitor, CMP: common myeloid progenitor, GMP: granulocyte macrophage progenitor, MEP: megakaryocyte erythrocyte progenitor. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. See also Supplementary Fig. 1-2.
ATF4 (HSPC: 2.1±0.1-fold, EP: 1.4±0.04-fold) and GADD34 (HSPC: 6.2±0.4-fold, EP: 1.8±0.2-fold) were more upregulated in HSPC compared to EP (Sup. Fig. 2b). In adult BM, upregulation of PERK pathway target genes was less dramatic, but CHOP expression was higher in HSPC compared to EP following addition of 3 μg/mL Tm (Sup. Fig. 2c). Thus, the selective enrichment of PERK pathway target genes under basal conditions in HSC is further amplified with Tm treatment.

Since persistent ER stress and UPR signaling can lead to activation of apoptosis through signals downstream of the IRE-1 and PERK branches of the UPR (Tabas and Ron, 2011), we wanted to determine whether differential UPR branch activation between HSPC and EP influenced cell fate outcome. While Tg treatment did not result in survival differences between HSPC and EP (Sup. Fig. 3a), Tm treatment significantly reduced CB-derived HSPC survival as compared to EP (HSPC: 33.3±4.1%, EP: 53.0±4.9% at 0.6 μg/mL Tm; HSPC: 2.1±0.4%, EP: 6.4±0.6% at 3 μg/mL Tm, Fig. 2a-b). Adult BM-derived HSPC also showed reduced survival at 3 μg/mL Tm (HSPC: 1.1±0.2%, EP: 16.1±4.4%, Fig. 2b). Cell cycle activation of CB-derived HSPC decreased but did not eliminate the survival difference with EP, suggesting that

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**Figure 2** | HSC are predisposed to apoptosis compared to EP following treatment with the ER stress agent Tm. a, b, Lower survival of CB- and BM-derived HSPC compared to EP in the presence of Tm. HSC/HSPC and EP were sorted from CB or adult BM and plated in the presence of (a) 0.6 μg/mL or (b) 3 μg/mL Tm. Viable cell counts as a percentage of DMSO controls are shown. Symbols represent individual samples of which fractions are connected by a black line, the blue line indicates mean±SEM of (a) n=16 CB and n=5 BM or (b) n=7 CB and n=5 BM. c, Reduced clonogenic potential of HSC compared to EP upon Tm treatment. 200 HSC or 140 EP were sorted into methylcellulose containing DMSO or 0.6 μg/mL Tm and colonies were counted 13 days later. Data is shown as mean±SEM of n=4 CB. d, e, Tm treatment causes higher apoptosis in HSC compared to EP. CB cells were plated with 0.6 μg/mL Tm. Cells were stained for primitive surface markers, Annexin-V and Sytox at different time points. (d) Representative flow plots show Annexin-V and Sytox staining. (e) Quantification of Annexin-V− cells is shown as mean±SEM of n=5 CB. p-values indicate different viability between HSC and EP. n.s.: not significant. * p<0.05, ** p<0.01, **** p<0.0001. See also Supplementary Fig. 3.
Tm sensitivity may be partly linked to the inherent quiescence of HSPCs (Sup. Fig. 3b). Tm reduced the clonogenic capacity of CD34⁺CD38⁺CD45RA⁻CD90⁺ HSC by 19-fold compared to 1.3-fold in EP (Fig. 2c, Sup. Fig. 3c-d). To determine if the selective loss of HSC following Tm treatment was due to apoptosis, we cultured CB cells with Tm and assessed apoptosis by Annexin-V staining. Although basal levels of viability were similar between HSC and EP, the remaining percentage of viable Annexin-V⁻ cells upon Tm treatment was lower for HSC compared to EP (HSC: 15±3.5%, EP: 47±6.1% after 40 hours, Fig. 2d-e). Increased apoptosis of HSC compared to EP following Tm treatment was confirmed using pre-sorted cells (Sup. Fig. S3c). Overall, these data indicate that Tm-induced ER stress not only elicits distinct UPR signaling in HSC compared to EP, but also causes selective apoptosis of HSC.

ER stress induces eIF2α phosphorylation (eIF2α–P) by PERK, leading to global translational attenuation but, paradoxically, ATF4 and CHOP translation is increased (Lu et al., 2004; Palam et al., 2011; Vattem and Wek, 2004). ATF4 and CHOP can induce apoptosis upon prolonged ER stress, in part by upregulating the eIF2α phosphatase GADD34, leading to increased protein load through translational recovery (Han et al., 2013; Marciniak et al., 2004). We investigated whether increased apoptosis of HSC compared to EP is linked to preferential upregulation of PERK pathway target genes. First, we constructed a lentiviral ATF4 reporter vector that marks transduced cells with TagBFP while GFP provides a measure for the ATF4 translation rate, which is increased when eIF2α–P is high due to the structure of the 5’ end of ATF4 mRNA (Lu et al., 2004) (Fig. 3a, Sup. Fig. 4). As expected, Tm treatment increased the ATF4 reporter transgene ratio (TGR) in transduced CB cells (2.47±0.17-fold), an effect that was inhibited by the PERK inhibitor GSK2606414 (Fig. 3b). In sorted HSPC and EP, the ATF4 reporter TGR was higher in HSPC compared to EP (Fig. 3c-d), consistent with increased baseline level ATF4 translation in HSPC. Following Tm addition, the TGR was more efficiently induced in HSPC compared to EP (HSC: 2.5±0.3 fold, p=0.032; EP: 1.9±0.2 fold, p=0.071, Fig. 3c-d), indicating that Tm stimulates PERK pathway activity more strongly in HSC compared to EP. Second, we over expressed constitutively active GADD34 (ca-GADD34-OE), which prevents eIF2α–P and upregulation of ATF4 and CHOP (Novoa et al., 2001). Expression of ca-GADD34 significantly increased HSPC survival following Tm treatment (CTRL: 41.0±5.7%, ca-GADD34-OE: 61.0±6.6% at 0.6 µg/mL Tm; CTRL: 2.8±0.8%, ca-GADD34-OE: 10.5±1.1% at 3 µg/mL Tm, Fig. 3e). In EP, the effect did not reach statistical significance (Fig. 3f), indicating that ca-GADD34-OE has a more pronounced effect on the stress response of HSC compared to EP. As a third method to test PERK pathway involvement, we treated sorted HSPC and EP with both Tm and Salubrinal (Sal), which prevents eIF2α dephosphorylation (Boyce et al., 2005). Without Sal addition, the viability of HSPC was lower than that of EP following Tm treatment (HSPC: 32±3%, EP: 56±2%, Fig. 3g). Addition of Sal and Tm together preferentially increased HSPC survival, resulting in similar viability between HSPC and EP (HSPC: 58±6%, EP: 64±2% at 20
Figure 3 | HSC are predisposed to UPR-induced apoptosis through PERK-eIF2α-ATF4-CHOP-GADD34 signaling. 

**a.** Bidirectional lentiviral reporter vector for ATF4 translation rate. All transduced cells are marked by TagBFP; GFP brightness is a measure of ATF4 mRNA translation, which is regulated by uORFs and depends on eIF2α~P (Lu et al., 2004). 

**b.** ATF4 reporter-transduced CB cells were treated with 0.6 µg/mL Tm and increasing doses of the PERK inhibitor GSK2606414. After 30 hours, the transgene ratio (TGR) between GFP and TagBFP was determined. Results are normalized to DMSO and shown as mean±SEM of n=6 CB except at 600 nM GSK2606414 (n=3). 

**c, d.** Higher ATF4 reporter activity in HSPC compared to EP, especially following Tm treatment. Sorted HSPC and EP were transduced with the ATF4 reporter. After 3 days, 0.6 µg/mL Tm was added to the cells and the TGR was measured 30 hours later. 

**e, f.** Over expression of constitutively active GADD34 (ca-GADD34 OE) has a more pronounced effect on HSPC compared to EP. 

**g, h.** Interfering with the PERK pathway at multiple junctions preferentially rescues HSC from apoptosis. Sorted HSPC and EP were plated with 0.6 µg/mL Tm. As well, (g) the GADD34 inhibitor Sal or (h) the PERK inhibitor GSK2606414 was added at concentrations indicated on the x-axes. The percentage of Annexin-V–Sytox− cells after 40 hours is shown as mean±SEM of (g) n=5 CB or (h) n=4 CB. uORF: upstream open reading frame, TGR: transgene ratio, MFI: mean fluorescence intensity. * p<0.05, ** p<0.01, *** p<0.001, **** p <0.0001. See also Supplementary Fig. 4.
μM Sal), indicating that the differential response between HSC and EP to Tm is dependent on GADD34-mediated dephosphorylation of eIF2α. Fourth, we treated sorted HSPC and EP with both Tm and the PERK inhibitor GSK2606414 (Axten et al., 2012). Like Sal, GSK2606414 reduced the difference in survival of Tm treatment between HSPC and EP (HSPC: 54.4±1.9%, EP: 58.7±2.5% at 600 nM GSK2606414, Fig. 3h). Thus, HSPC can be protected from Tm-induced apoptosis by interfering with the PERK pathway at multiple junctions, and this equalizes the survival between HSPC and EP. Collectively, these data demonstrate that Tm-induced ER stress preferentially induces signaling through the PERK branch of the UPR in HSC compared to EP, resulting in selective upregulation of pro-apoptotic genes and increased HSC death.

We next asked whether the UPR was directly involved in regulating HSC function under physiological stress conditions. Since CHOP is a main driver of apoptosis upon PERK activation (Marciniak et al., 2004; Tabas and Ron, 2011), the BM of Chop−/− mice was analyzed. The frequency of mouse stem and progenitor cells was not significantly changed in Chop−/− mice compared to wild-type controls, but there was a small increase in the basal levels of viability of Lin−Sca-1+c-Kit+ mouse HSC-enriched cells as well as Lin−Sca-1−c-Kit+ progenitors (Sup. Fig. 5a-b, Sup. Table 3). This suggests that Chop may be required for the survival/death balance of mouse hematopoietic progenitors under physiological conditions. Next, we investigated whether modulating UPR signaling would alter human HSC function. The UPR target gene ERDJ4 is a co-chaperone that increases the ATPase activity of GRP78 and can be released from the ER membrane to associate with the ERAD machinery (Lai et al., 2012; Shen et al., 2002). These functions may enhance cellular protein folding capacity and protect against UPR-induced apoptosis (Kurisu et al., 2003). ERDJ4 expression was highest in purified CB-derived CD49f+ HSC and was reduced in CD49f+ multipotent progenitors (2.0-fold) as well as all EP fractions (2.2- to 4.1-fold, Fig. 4a). GFP-marked ERDJ4 over expression (ERDJ4-OE) lentiviral vectors were constructed to express different transgene levels (2.3× with PGK-ERDJ4 and 218× with SFFV-ERDJ4, Fig. 4b-c). In the TEX cell line (Warner et al., 2005), SFFV-ERDJ4 expression decreased Tm-induced apoptosis (Sup. Fig. 5c-d). Importantly, CB-derived HSPC expressing SFFV-ERDJ4 were also protected from Tm-induced cell death (SFFV-CTRL: 51±4% survival, SFFV-ERDJ4: 62±5% survival, Fig. 4d), suggesting that ERDJ4-OE increases the threshold of ER stress needed to induce apoptosis. To test whether ERDJ4 influences human HSC function, CB cells were transduced with PGK-ERDJ4 and transplanted into immune-deficient mice. After 20 weeks of in vivo competition between GFP+ with GFP− cells the percentage of GFP+ cells in the PGK-ERDJ4 group was increased compared to the PGK-CTRL group (PGK-CTRL day 0: 28±3.8%, 20 weeks: 27±5.5%; PGK-ERDJ4 day 0: 44±9.4%, 20 weeks: 70±7.9%; Fig. 4e, Sup. Fig. 5e). To directly measure the impact of ERDJ4-OE on the function and number of HSC, in vivo limiting dilution analysis (LDA) was performed. PGK-ERDJ4 transduced CB
cells were expanded in vitro and sorted GFP+ cells were injected at limiting cell doses, resulting in higher engraftment compared to CTRL cells (Fig. 4f, Sup. Fig. 5f-g). The number of mice with human engraftment was also changed: at the low dose, 5 out of 11 control mice were engrafted, compared to 11 out of 12 ERDJ4-OE mice, demonstrating by LDA that there was a 4.4-fold increase in the number of repopulating HSC in the ERDJ4-OE group (CTRL: 1/73137, ERDJ4-OE: 1/16641, Fig. 4g). These data provide strong evidence that a protein folding factor class-
cally associated with the UPR governs HSC function.

To understand the mechanism by which ERDJ4-OE can improve human HSC function, we confirmed that ERDJ4-OE does not change in vivo lineage differentiation, the frequency of phenotypic stem and progenitor cell compartments, homing, or progenitor cell engraftment (Sup. Fig. 6a-d). This suggests that ERDJ4-OE does not have a strong effect on progenitor cells. As well, secondary LDA showed that ERDJ4-OE does not cause an expansion of functional HSC during repopulation (Sup. Fig. 6e), indicating that the effects of ERDJ4-OE are transient. To investigate whether ERDJ4-OE protects against ER stress during the in vivo transplantation procedure, CHOP and GADD34 expression was quantified at different time points. In PGK-CTRL transduced CB cells, induction of CHOP (2.2±0.6-fold) and GADD34 (14.4±4.0-fold) expression was seen 19 hours after transplantation compared to before transplantation (Fig. 4h), consistent with a stress response upon transplantation. In contrast, in PGK-ERDJ4 transduced CB cells, this surge in CHOP and GADD34 expression was absent (0.82±0.18-fold and 0.98±0.06-fold, respectively). These data suggest that ERDJ4-OE improves engraftment by protecting HSC from UPR-induced apoptosis following transplantation. Transplantation of human HSC in the xenograft environment places them under replicative stress, which causes elevated ROS, DNA damage, and loss of HSC function (Yahata et al., 2011). Since both ROS and DNA damage can cause ER stress, it is possible that these processes are connected to the UPR (Rutkowski and Kaufman, 2004). In support of this idea, it was recently shown that ROS accumulation leads to UPR-mediated apoptosis of HSC (Rouault-Pierre et al., 2013). Our findings implicate ER proteostasis as a critical regulator of HSC function during repopulation and suggest that moderation of UPR activation may have clinical applications if it improves HSC survival during stem cell transplantation.

Our results establish a previously unrecognized link between UPR signaling and human HSC function. The different branches of the UPR are distinctly activated in HSC compared to EP, with the consequence that HSC are rapidly cleared while EP are spared when exposed to various sources of stress. This biological response of human HSC is consistent with the HSC specific induction of apoptosis seen following DNA damage and ROS accumulation (Ito et al., 2004; Milyavsky et al., 2010; Yahata et al., 2011). Collectively, these data reveal an intrinsic biological focus on preventing propagation of damaged HSC that would increase the chance of malignancy. Since terminal differentiation effectively purges damaged progenitor cell progeny, clonal purity may be of less importance in EP. Loss of HSC and intestinal stem cells in mouse models with deletion of the chaperone Grp78 support the idea that stem cells of multiple tissues can interrogate ER stress, and utilize differential UPR activation to mitigate against potentially pathological damage (Heijmans et al., 2013; Wey et al., 2012). Overall, our data point to the elimination of individual HSC following stress and damage as a paradigm of how the stem cell pool maintains integrity, thereby ensuring long-term tissue maintenance.
4.3 Methods summary
CB cells were lineage depleted to enrich for CD34+ cells and then sorted by Fluorescence Associated Cell Sorting, cultured with ER stress-inducing agents, and/or transduced with lentivirus. Quantitative RT-PCR was performed using a 7900 HT Real-Time PCR system, primer sequences are listed in Sup. Table 2. Apoptosis was assessed by Annexin-V/Sytox or cleaved Caspase-3 staining followed by flow cytometry. Intrafemoral transplantation of human CB cells into immune-deficient mice was used to read out HSC repopulation activity by flow cytometry after 10-20 weeks. Unless otherwise stated, p-values were calculated by two-tailed unpaired Student’s t-test. Full Methods are provided in the Supplementary Information.

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4.5 References


