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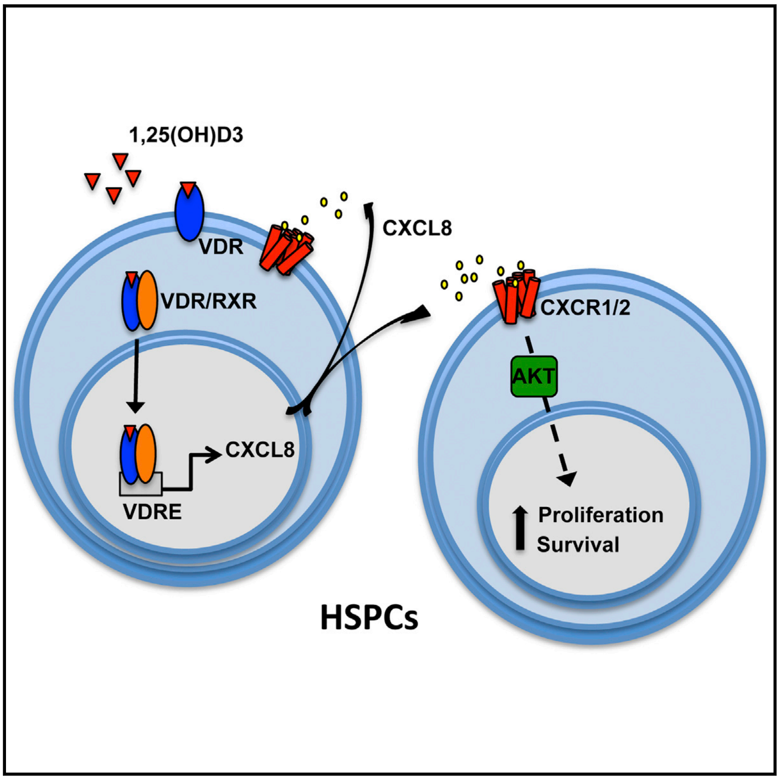
Developmental Vitamin D Availability Impacts Hematopoietic Stem Cell Production

Graphical Abstract



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In Brief

Cortes et al. elucidate a role for vitamin D signaling in hematopoietic stem and progenitor (HSPC) expansion and survival. Using zebrafish embryos and human umbilical cord blood, they demonstrate that HSPCs respond directly to 1,25(OH)D3 stimulation via vitamin D receptor-induced transcriptional activation of the inflammatory cytokine CXCL8.

Highlights

- Developmental 1,25(OH)D3 availability modulates definitive HSPC production
- The effect of 1,25(OH)D3 on HSPCs is VDR-mediated and Ca^{2+}-independent
- Vitamin D supplementation significantly increases human UCB HSPCs in vitro
- 1,25(OH)D3 exposure promotes viability and proliferation via Cxcl8 (IL-8) activity

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Developmental Vitamin D Availability Impacts Hematopoietic Stem Cell Production

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SUMMARY

Vitamin D insufficiency is a worldwide epidemic affecting billions of individuals, including pregnant women and children. Despite its high incidence, the impact of active vitamin D3 (1,25(OH)D3) on embryonic development beyond osteo-regulation remains largely undefined. Here, we demonstrate that 1,25(OH)D3 availability modulates zebrafish hematopoietic stem and progenitor cell (HSPC) production. Loss of Cyp27b1-mediated biosynthesis or vitamin D receptor (VDR) function by gene knockdown resulted in significantly reduced *runx1* expression and Flk1$^+$cMyb$^+$ HSPC numbers. Selective modulation in vivo and in vitro in zebrafish indicated that vitamin D3 acts directly on HSPCs, independent of calcium regulation, to increase proliferation. Notably, ex vivo treatment of human HSPCs with 1,25(OH)D3 also enhanced hematopoietic colony numbers, illustrating conservation across species. Finally, gene expression and epistasis analysis indicated that *CXCL8* (*IL-8*) was a functional target of vitamin D3-mediated HSPC regulation. Together, these findings highlight the relevance of developmental 1,25(OH)D3 availability for definitive hematopoiesis and suggest potential therapeutic utility in HSPC expansion.

INTRODUCTION

Characterizing the impact of environmental modifiers on hematopoietic stem and progenitor cell (HSPC) formation and maintenance is of significant therapeutic interest because these extrinsic factors may influence both the onset and outcome of hematological disease. Active vitamin D (1,25(OH)D3), a fat-soluble hormone required for many physiological functions, is derived via sunlight and diet in humans. As such, factors including geographical location, lifestyle, and nutritional regimen can impact the level of vitamin D sufficiency. Recent studies indicate that vitamin D insufficiency is now a global epidemic, affecting over 1 billion people (Naeem, 2010). Among pregnant women, the worldwide incidence of 1,25(OH)D3 deficiency is estimated to be between 6% and 96% depending on geographical location and availability of supplementation (Weinert and Silveiro, 2015), with a particularly high occurrence in industrialized regions (40%–50%) (Bodnar et al., 2007). Severe 1,25(OH)D3 deficiency because of biosynthetic or signaling pathway mutations results in insufficient calcium (Ca^{2+}) absorption and abnormal bone mineralization, leading to rickets in children and osteomalacia in adults. Interestingly, case studies of children with severe vitamin D deficiency present a variety of hematological disorders (Holick, 2006; Yetgin and Ozsoylu, 1982); however, the impact of vitamin D status on embryonic hematopoietic stem cell (HSC) production has not been elucidated.

De novo vitamin D synthesis begins with transformation of 7-dehydrocholesterol in the skin by UV radiation to the non-active vitamin D precursor cholecalciferol (D3). D3 is then modified by cytochrome P450 enzyme 2R1 (CYP2R1), primarily in the liver, to generate 25-hydroxy vitamin D (25(OH)D3, calcidiol), the circulating form of vitamin D. In the kidney, 25(OH)D3 is further hydroxylated by CYP27B1 to generate the active metabolite 1,25-dihydroxy vitamin D (1,25(OH)D3, calcitriol). 1,25(OH)D3 acts as a ligand for the vitamin D receptor (VDR), a member of the nuclear hormone receptor family (Mizwicki and Norman, 2009). Upon ligand binding, VDR forms a heterodimer with the retinoic acid X receptor (RXR), resulting in nuclear localization and transcriptional regulation of genes with vitamin D response elements (VDREs) (Plum and DeLuca, 2010). Genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) studies identified more than 200 VDR-regulated genes (Ramagopalan et al., 2010). 1,25(OH)D3/VDR binding can also elicit non-genomic responses, including extracellular Ca^{2+} uptake (Norman, 2006).

In humans, mutations in genes of the vitamin D biosynthetic pathway are the most common cause of vitamin D-dependent rickets (VDDR), whereas VDR mutations result in hereditary vitamin D-resistant rickets (HVDR) (Holick, 2006). VDDR is treated with vitamin D supplementation, whereas HVDR is partially ameliorated with supplemental Ca^{2+}. Children with vitamin D deficiency-associated rickets are clinically documented to exhibit a variety of hematopoietic defects, including anemia, extramedullary hematopoiesis, thrombocytopenia, myelofibrosis, and myelodysplasia. Some features improve with Ca^{2+} supplementation, suggestive of bone marrow (BM) niche dysregulation (Yetgin and Ozsoylu, 1982). Indeed, *VDR* loss in the mouse leads to extramedullary hematopoiesis because of abnormal bone mineralization (Jeanson and Scadden, 2010). In contrast, expression analyses indicate that VDR is one of 33 genes highly enriched in mouse HSCs (Riddell et al., 2014). VDR levels vary as immune cells mature, with monocytes/macrophages showing downregulation (Hewison et al., 2003) and T cells upregulation, respectively (Bruce and Cantorna, 2011). Subsets of T cells also express *Cyp27b1*, implying that local 1,25(OH)D3 synthesis may be important for T cell function (Ooi et al., 2014). Further, *Cyp27b1*-null mice show reductions in CD4 and CD8 T cells (Panda et al., 2001). Similar to mice, *VDR* is broadly expressed in human hematopoietic cells (Kizaki et al., 1991). Children with von Jaksch-Luzet syndrome, a severe vitamin D deficiency, are characterized by anemia with accumulation of erythroblasts and myeloblasts resembling chronic myeloid leukemia (Holick, 2006; Kassam et al., 1983). Despite multi-lineage abnormalities and frequent childhood onset, minimal investigation has been made into the impact of vitamin D status on developmental HSPC production.

Unlike adult HSPC homeostasis, which is strongly regulated by interactions with the BM microenvironment, embryonic hematopoiesis is initiated de novo in specialized vascular niches with transient hematopoietic potential (Carroll and North, 2014). Here we present a role for active vitamin D3 in embryonic HSPC production: 1,25(OH)D3 levels directly impact HSPC numbers in vivo and in vitro via VDR-mediated regulation of pro-proliferative responses independent of Ca^{2+} flux. Significantly, reduction in 1,25(OH)D3 production or function because of perturbations in biosynthetic or signaling genes decreased HSPCs in zebrafish embryos. In contrast, 1,25(OH)D3 supplementation expanded HSPCs in vivo and in vitro. Gene expression and epistasis studies indicated that 1,25(OH)D3 regulates *cxcl8* (*IL-8*) signaling to control HSPC numbers. Finally, ex vivo treatment with 1,25(OH)D3 elicited significant elevations in survival, proliferation, and multi-lineage colony forming activity of $CD34^{+}$ human umbilical cord blood HSCs. Together, these data highlight the conservation of 1,25(OH)D3 function in developmental HSC regulation and indicate the potential utility of 1,25(OH)D3 supplementation for HSPC expansion in clinical transplantation therapies.

RESULTS

VDR Signaling Is Essential for Hematopoietic Stem and Progenitor Cell Production

Despite case studies of hematological problems in children with severe 1,25(OH)D3 deficiency, the effects on embryonic hematopoiesis remain uncharacterized. Vitamin D was identified as a modulator of HSPC expression in a prior compound screen (North et al., 2007). We recently showed that accumulation of the inactive vitamin D precursor, cholecalciferol, negatively affected hemogenic endothelial formation, independent of VDR activation, by directly antagonizing Hedgehog signaling (Cortes et al., 2015). To investigate whether active 1,25(OH)D3-associated VDR signaling impacted definitive hematopoiesis, we examined the effect of *VDR* loss on HSPC development. The zebrafish genome encodes two VDRs, *vdra* and *vdrb*, with *vdra* alone acting as the canonical VDR in vivo (Lin et al., 2012). Fractions from Tg(*flk1:dsRed;cmyb:GFP)* embryos (1,000 embryos/sample × 3 replicate sorts) isolated by fluorescence-activated cell sorting (FACS) showed *vdra* expression in endothelial niche cells ($Flk1{:}dsRed^{+}/cMyb{:}GFP^{-}$) and HSPCs ($Flk1{:}dsRed^{+}/cMyb{:}GFP^{+}$) (Figure S1A). Morpholino (MO) knockdown of *vdra* strongly reduced *runx1* expression in the aorta-gonad-mesonephros (AGM) region 36 hr post fertilization (hpf) by whole-mount in situ hybridization (WISH) (Figure 1A) without overt defects in the vascular niche, as marked by *flk1* (Figure 1B) and *ephrinb2* (Figure S1B). This result was confirmed and quantified by qPCR analysis (*runx1*, $p < 0.01$; *flk1*, p = not significant [N.S.]; *ephrinb2a*, p = N.S.) (Figure 1C). In contrast, *vdrb* knockdown had no effect on *runx1* (Figure S1C). The specificity of the *vdra* MO was confirmed by splice product detection (Figure S1E) and rescued by addition of *vdra*, but not *vdrb*, mRNA (Figure S1F). Importantly, generation of a *vdra* mutant embryo by CRISPR-Cas9 genome editing (Figures S1G and S1H) resulted in the same phenotypic effects on both *runx1* (decreased) and *flk1* (unchanged) by WISH and qPCR (*runx1*, $p < 0.01$; *flk1*, p = N.S.; *ephrinb2*, p = N.S.) (Figures 1D–1F). Whole-embryo FACS quantification performed with the Tg(*lmo2:GFP;gata1:dsRed)* line to mark functional erythro-myeloid progenitors (EMPs) (Bertrand et al., 2007) and Tg(*flk1:dsRed;cmyb:GFP)* to mark HSPCs (Bertrand et al., 2010) indicated a 20% ($p < 0.05$) decrease at 30 hpf (Figure S1D) and 38% ($p < 0.05$) reduction by 48 hpf (Figure 1G), respectively, with *vdra* knockdown. Further, *vdra* morphants had sustained reductions in $CD41{:}GFP^{+}$ HSCs colonizing the caudal hematopoietic tissue (CHT) at 72 hpf in the Tg(*-6.0itga2b(CD41):eGFP*) line, as determined by fluorescence microscopy (Figure S1I) and FACS (Figure S1J; $p < 0.01$). To confirm that the levels of vitamin D3/VDR activity correlated with embryonic HSPC output, the impact of reduced vitamin D synthesis was also examined. MO knockdown of *cyp27b1*, which controls the final hydroxylation step in 1,25(OH)D3 synthesis, diminished *runx1/cmyb* expression (Figure 1H). FACS analysis of *cyp27b1* morphants confirmed a significant decrease ($p < 0.01$) in HSPCs (Figure 1I), consistent with a role for 1,25(OH)D3/VDR activity in embryonic HSPC production.

Vitamin D Supplementation Stimulates Embryonic HSPC Production

Because vitamin D synthesis and signaling appeared to be necessary for the establishment of normal embryonic HSPC numbers, we next examined whether supplementation was sufficient to stimulate production. We previously determined that *cyp27b1* is expressed by 12 hpf (Cortes et al., 2015). Exposure to exogenous 25(OH)D3 (10 μM), the circulating form of vitamin D, enhanced *runx1/cmyb* expression (Figure 2A), which was confirmed and quantified as a 2-fold increase in $Flk1^{+}/cMyb^{+}$ HSPCs by FACS ($p < 0.01$; Figure 2B). Consistent with biosynthetic processing by Cyp27b1 and VDR activation,

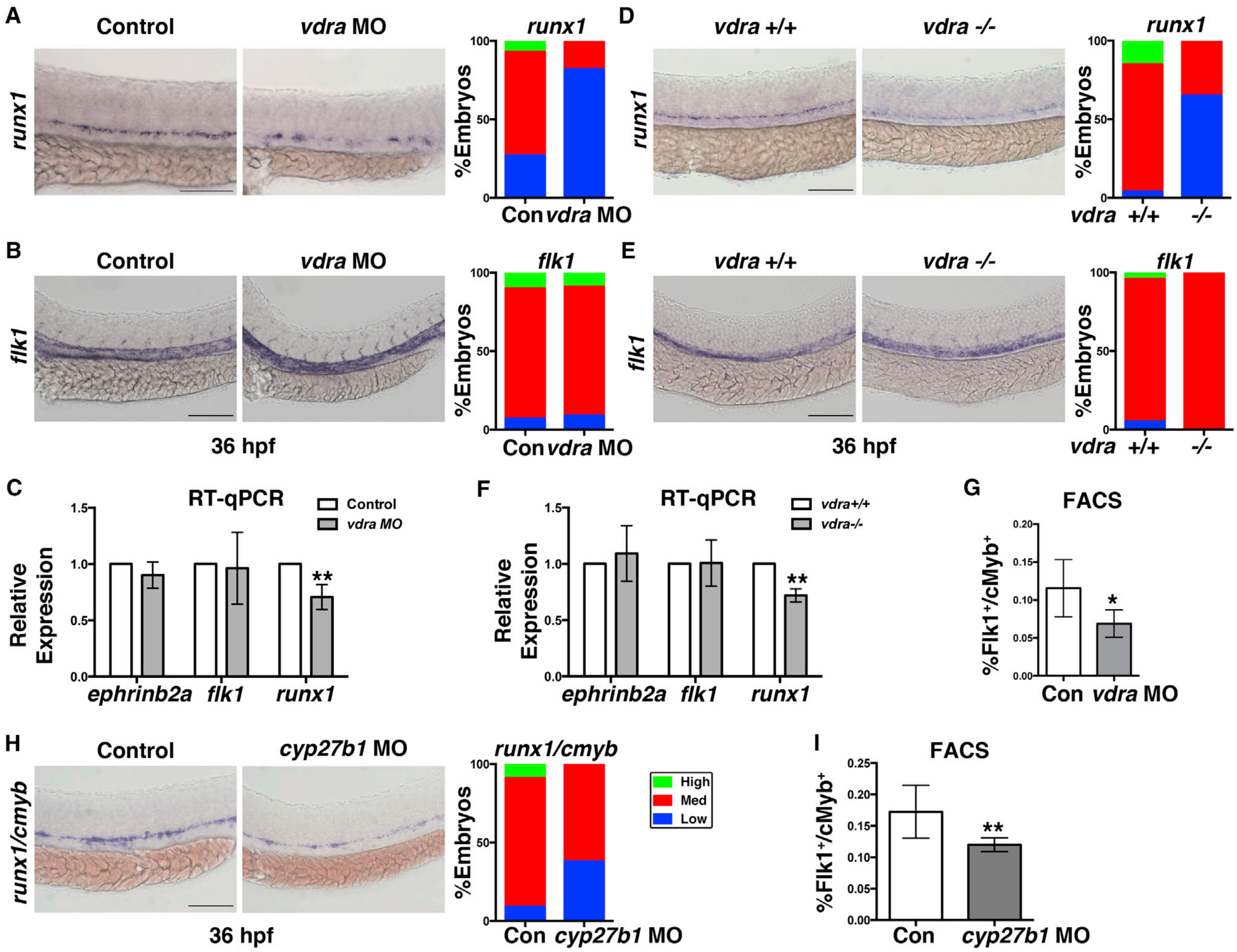


Figure 1. VDR Is Necessary for Zebrafish Definitive Hematopoiesis

(A) Representative *runx1* WISH images and qualitative phenotype distribution (high/medium/low, n $\geq$ 30 embryos/condition) of *vdra* MO-injected embryos and sibling controls at 36 hpf. Error bars, mean $\pm$ SD.

(B) WISH example and phenotype distribution for *flk1* in *vdra* morphants (n $\geq$ 20 embryos/condition).

(C) qPCR analysis quantifying the impact on HSPCs (*runx1*, **p < 0.01) and hemogenic endothelium (*flk1*, *ephrinb2a*, p = N.S.) in control and *vdra* morphants (30 embryos/sample $\times$ 3 replicates).

(D–F) Representative WISH (D), phenotype distributions (E), and qPCR quantification (F) of *vdra* mutant embryos (*runx1*, **p < 0.01; *flk1*, *ephrinb2a*, p = N.S.) phenocopied the impact on HSPCs but not on vessels.

(G) FACS analysis of *vdra* morphants indicated significant reductions in Flk1:dsRed$^+$/cMyb:GFP$^+$ HSPCs (*p < 0.05) at 48 hpf (5 embryos/sample $\times$ 4 replicates/condition). Error bars, mean $\pm$ SD.

(H) WISH samples and phenotype distribution of *runx1/cmyb* expression in *cyp27b1* MO-injected embryos at 36 hpf (n $\geq$ 30 embryos/condition).

(I) FACS analysis showed a significant decrease in HSPCs (**p < 0.01) with *cyp27b1* MO injection (n value and error bars as in C).

Scale bars, 100 μm. See also Figure S1.

25(OH)D3-treated embryos had elevated expression of the VDR target *cyp24a1* by qPCR at 36 hpf (p < 0.05), similar to 1,25(OH)D3 (p < 0.001; Figure 2C). VDR activation was confirmed using a Tg(*VDRE:mCherry*) reporter line that showed enhanced *mCherry* expression in the AGM with both 25(OH)D3 and 1,25(OH)D3 exposure (Figure S2A). Likewise, FACS indicated a significant increase in Flk1:GPF$^+$/VDRE:mCherry$^+$ cells with 1,25(OH)D3 (p < 0.0001; Figure S2B). To further examine correlations between active vitamin D3 levels and HSPC production, dosage analysis was performed. A dose-responsive induction in the distribution of embryos with high *runx1/cmyb* expression was seen with 0.1–10 μM 1,25(OH)D3 exposure (Figure 2D). FACS quantification further revealed that exposure to 10 μM 1,25(OH)D3 caused a 25% (p < 0.01) increase in the number of EMPs at 30 hpf (Figure S2C) and a 20% (p < 0.01) increase in AGM-derived HSPCs at 48 hpf (Figure 2E). Together with enhanced HSPC production, a 2-fold increase in proliferation in the AGM was observed with 1,25(OH)D3 treatment (p < 0.001) compared with controls (Figure S2D) using the fluorescence ubiquitination cell cycle indicator (FUCCI) reporter

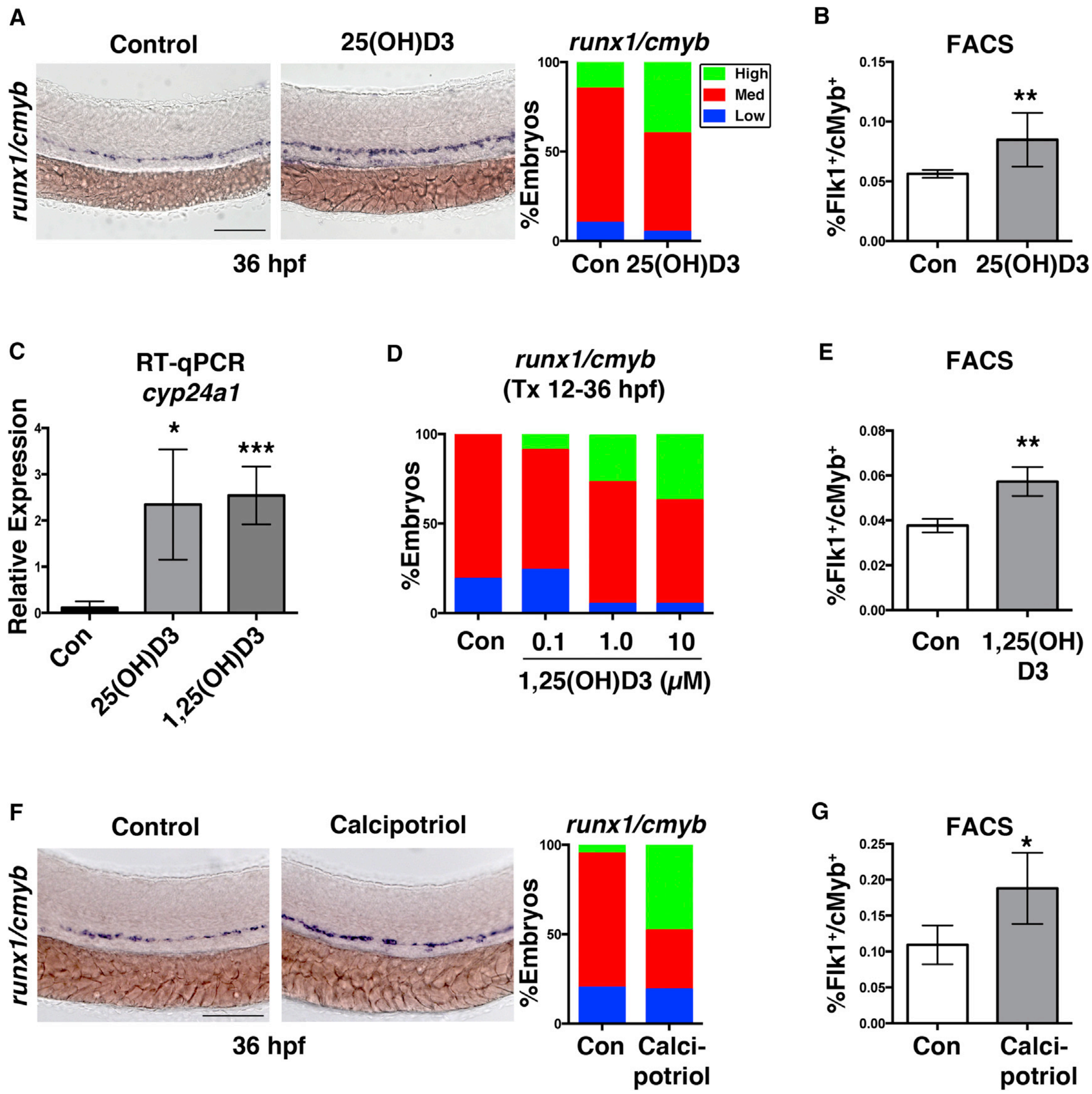


Figure 2. 1,25(OH)D3 Regulates HSPC Numbers via Vitamin D Receptor Signaling

(A) Representative *runx1/cmyb* WISH images and qualitative phenotype distribution following 25(OH)D3 exposure from 12–36 hpf ($n \geq 40$ embryos/condition).

(B) FACS analysis of 25(OH)D3-treated (10 μM) embryos showed a significant increase in HSPCs (**$p < 0.01$) (5 embryos/sample × 4 replicates/condition). Error bars, mean ± SD.

(C) qPCR analysis indicated a significant induction in the VDR transcriptional target *cyp24a1* at 36 hpf after exposure to 25(OH)D3 (*$p < 0.05$) or 1,25(OH)D3 (***$p < 0.001$) (12–36 hpf) (30 pooled embryos/condition × 3 replicates).

(D) Phenotype distribution of *runx1/cmyb* expression in embryos exposed to increasing concentrations of 1,25(OH)D3 from 12–36 hpf ($n \geq 30$ embryos/condition).

(E) FACS analysis of 1,25(OH)D3-treated (10 μM) embryos showed a 20% increase in HSPCs at 48 hpf (**$p < 0.01$) compared with controls (n value and error bars as in B).

(F) *runx1/cmyb* WISH and phenotype distribution after exposure to the non-calcemic vitamin D3 analog calcipotriol ($n \geq 30$ embryos/condition).

(G) FACS analysis of calcipotriol-treated embryos showed a significantly more HSPCs (*$p < 0.05$) at 36 hpf (n value and error bars as in B).

Scale bars, 100 μm. See also Figure S2.

Tg(EF1:mAG-zGEM(1/100) line (Sugiyama et al., 2009) crossed to *Tg(flk1:dsred)*. In contrast, *vdra* knockdown caused a significant decrease in proliferation ($p < 0.05$; Figure S2E). Finally, to determine whether the effect of 1,25(OH)D3 was due to VDR-mediated transcriptional regulation or alterations in Ca^{2+} flux, embryos were treated with the vitamin D3 analog calcipotriol, documented to be 100-fold less calcemic than 1,25(OH)D3 (Binderup and Bramm, 1988). Calcipotriol (10 μM) shifted the distribution of embryos with increased *runx1/cmyb* expression in a manner phenotypically comparable with 1,25(OH)D3 (Figure 2F) and caused a 29% elevation in $Flk1^+cMyb^+$ HSPCs ($p < 0.05$) by FACS (Figure 2G). Furthermore, the impact of both 1,25(OH)D3

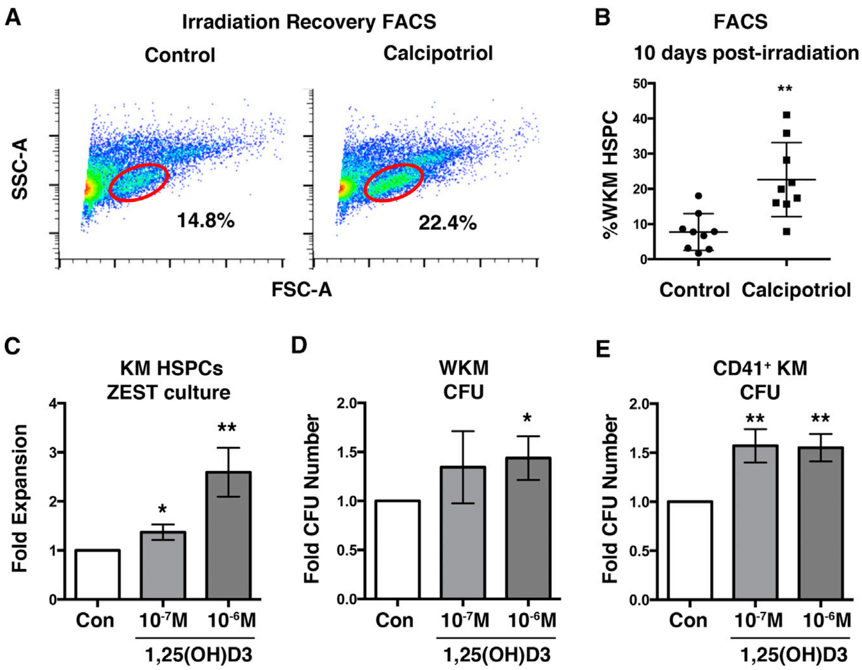


Figure 3. 1,25(OH)D3 Stimulates Cell-Autonomous HSPC Expansion

(A) Representative FACS plots of DMSO- and calcipotriol-treated (20 μM) adult zebrafish at 10 dpi showing that calcipotriol enhanced the percentage of FSC/SSC-gated HSPCs (n = 9 adults/condition).

(B) Calcipotriol-treated adults had a 34% (**$p < 0.01$) increase in HSPCs compared with controls at 10 dpi (error bars, mean ± SD) by FACS.

(C) Adult KM HSPCs cultured with 1,25(OH)D3 for 6 days on ZEST cells demonstrated increased proliferation (**$p < 0.01$) compared with controls (n = 3). Error bars, mean ± SD.

(D) Methylcellulose CFU assay of adult whole KM (WKM) cells cultured with of 1,25(OH)D3 (1×10^{-7} M) displayed a 40% (*$p < 0.05$) increase in total colonies compared with DMSO controls (n = 3). Error bars, mean ± SD.

(E) CFU assay of CD41:GFP$^+$ KM cultured in the presence of 1×10^{-6} and 1×10^{-7} M 1,25(OH)D3 and sorted by FACS showed a 55% (**$p < 0.01$) and 57% (**$p < 0.01$) elevation, respectively, in colony numbers versus controls (n = 3). Error bars, mean ± SD.

See also Figure S3.

and calcipotriol was sustained in the CHT at 72 hpf, as determined by microscopy and FACS for CD41:GFP$^+$ (control [con] versus 1,25(OH)D3, $p < 0.05$; con versus calcipotriol, $p < 0.01$) (Figures S2F and S2G). Together, these data indicate that vitamin D3-mediated regulation of embryonic HSPC production occurs via stimulation of VDR activity and cell proliferation independent of impact on Ca^{2+} regulation.

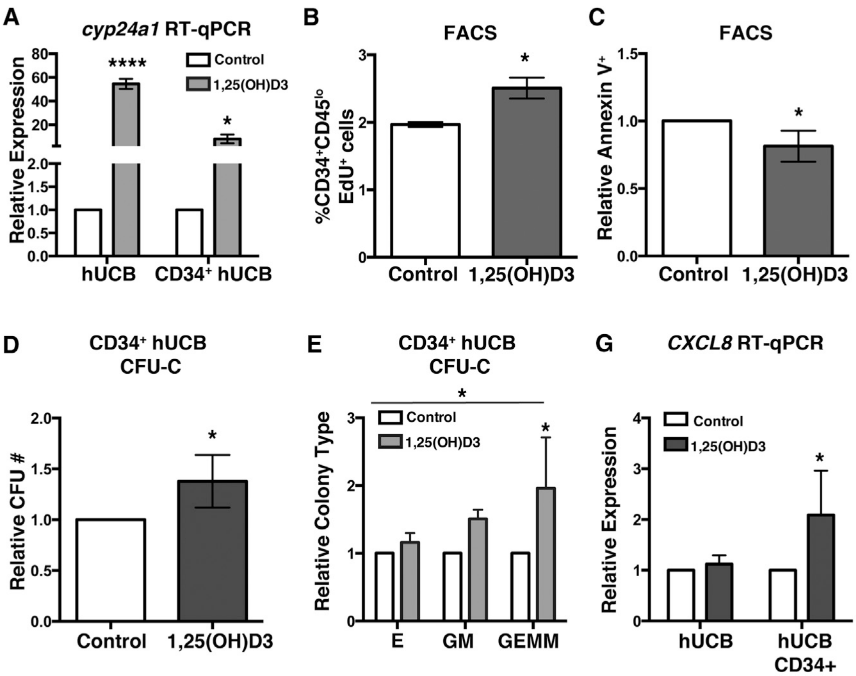
1,25(OH)D3 Increases Adult HSPC Production and Function In Vivo and In Vitro

Unlike the mammalian BM niche, which is directly affected by vitamin D/VDR-mediated Ca^{2+} flux, adult zebrafish kidney marrow (KM) allows for analysis of 1,25(OH)D3 function independent of osteoregulation. To determine whether embryonic modulation of vitamin D production or signaling had long-term consequences, KM was dissected and subjected to FACS forward scatter (FSC)/side scatter (SSC) profiling (Stachura and Traver, 2016). KM from zebrafish exposed to exogenous 1,25(OH)D3 transiently as embryos (12–36 hpf) did not differ from control siblings (Figure S3A). In contrast, both *cyp2r1* and *vdra* mutants showed significant reductions in cells within the "lymphoid" gate ($p < 0.05$), which includes both lymphoid progenitors and HSCs (Stachura and Traver, 2016), implying that vitamin D sufficiency is necessary to establish normal HSPC numbers (Figures S3B and S3C).

To investigate whether 1,25(OH)D3 supplementation could stimulate HSPC production in the adult, KM irradiation recovery experiments were performed (North et al., 2007). Consistent with adverse effects observed in the clinic (Leyssens et al., 2014), systemic treatment with 1,25(OH)D3 (10 μM) following irradiation was toxic, likely because of hypercalcemia, precluding further analysis. In contrast, exposure to calcipotriol for 24 hr at 2 days post sub-lethal irradiation (dpi) caused a significant 34% enhancement ($p < 0.01$) in the recovery of HSPCs at 10 dpi compared with DMSO-treated controls (Figures 3A and 3B). To further characterize the impact of vitamin D3 supplementation, manually isolated whole KM was cultured on either zebrafish embryonic stromal trunk (ZEST) cells or zebrafish kidney stromal (ZKS) cells (Campbell et al., 2015). After 6 days in culture, a significant increase in total hematopoietic cells was observed with 1,25(OH)D3 exposure (ZEST, $p < 0.01$; ZKS, $p < 0.01$) (Figure 3C; Figure S3D), indicative of pro-proliferative stimulation. To determine whether 1,25(OH)D3 had a direct impact on HSPC function, methylcellulose hematopoietic colony-forming assays were performed (Stachura et al., 2011). Dissociated KM plated in the presence of 1,25(OH)D3 (1×10^{-6} M) showed a 40% increase ($p < 0.05$) in total colony forming units, culture (CFU-C) compared with DMSO controls (Figure 3D) on day 10 after plating. Significantly, FACS-sorted CD41:GFP$^+$ KM HSPCs cultured with 1,25(OH)D3 displayed a similar expansion (1×10^{-6} M, 55% increase, $p < 0.01$; 1×10^{-7} M, 57% increase, $p < 0.01$) (Figure 3E), indicating that adult HSPCs can respond cell-autonomously to vitamin D3 stimulation to boost HSPC production and function, independent of osteoregulation.

1,25(OH)D3 Enhances In Vitro Function of CD34$^+$ Human Umbilical Cord Blood

Given the strong pro-HSPC expansion effect observed in vivo and in vitro in zebrafish, we next investigated whether the regulatory impact of 1,25(OH)D3 was conserved in mammalian HSPCs. VDR is expressed on highly enriched murine HSCs (Riddell et al., 2014) as well as human CD34$^+$ hematopoietic progenitors (Grande et al., 2002). Expression of the human *VDR* was confirmed in commercially available de-identified whole (unfractionated) and CD34$^+$ human umbilical cord blood (hUCB) by qPCR (Figure S4A). Ex vivo treatment with 1,25(OH)D3 for 4 hr



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Ingenuity Canonical Pathways	-log(p-value)
RAN Signaling	5.99
Protein Ubiquitination Pathway	4.79
EIF2 Signaling	4.77
Cleavage and Polyadenylation of Pre-mRNA	3.44
Oxidative Phosphorylation	3.42
Mitochondrial Dysfunction	2.91
Regulation of eIF4 and p70S6K Signaling	2.67
VEGF Signaling	2.27
IL-8 Signaling	2.12
Interferon Signaling	2.02

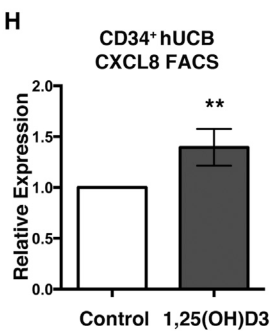

Figure 4. Ex Vivo 1,25(OH)D3 Exposure Increases hUCB CD34$^+$ HSPCs

(A) qPCR analysis for the known VDR target *CYP24A1* showed increased expression (****$p < 0.0001$) in individual whole and CD34$^+$ (*$p < 0.05$) hUCB units split and treated with 1,25(OH)D3 versus DMSO (vehicle) control (n = 3 hUCB units). Error bars, mean ± SD.

(B) Whole (umbilical) cord blood (WCB) cells treated with 1,25(OH)D3 (4 hr) had significantly increased EdU labeling (*$p < 0.05$) of CD34$^+$CD45^{lo} HSCs compared with matched controls (n = 2). Error bars, mean ± SD.

(C) Annexin-V/7-AAD FACS analysis of CD34$^+$ hUCB showed a significant decrease (*$p < 0.05$, n = 3) in apoptosis with 1,25(OH)D3 treatment.

(D) In vitro methylcellulose assays of CD34$^+$ hUCB treated ex vivo with 1,25(OH)D3 (1×10^{-6} M) for 4 hr revealed a 38% (*$p < 0.05$) increase in total CFU numbers compared with DMSO controls on day 7 after plating (five independent single donor hUCB samples split for treatment and analysis). Error bars, mean ± SD.

(E) 1,25(OH)D3 treatment enhanced all colony types (CFU-E, CFU-GM, and CFU-GEMM) on day 10 versus matched controls (ANOVA, *$p < 0.05$), with a significant increase in GEMMs (Fisher's least significant difference [LSD] test, *$p < 0.05$). n = 5; error bars, ± SEM.

(F) Top canonical pathways enriched by 1,25(OH)D3 exposure based on IPA of significantly expressed genes. False discovery rate (FDR) < 0.05 (n = 2 single donor CD34$^+$ hUCBs); IL-8 (CXCL8). Signaling is highlighted in bold type.

(G) qPCR analysis of CD34$^+$ hUCB cells exposed to 1,25(OH)D3 (4 hr) showed a significant increase in *CXCL8* expression (*$p < 0.05$) (n = 3). Error bars, mean ± SD.

(H) FACS quantification for CXCL8 protein content in CD34$^+$ hUCBs showed a significant increase with 1,25(OH)D3 treatment (**$p < 0.01$ n = 4; error bars, mean ± SD).

See also Figure S4.

led to significant upregulation of *CYP24A1* in both whole ($p < 0.0001$) and CD34$^+$ ($p < 0.05$) hUCB cells (Figure 4A), indicative of VDR activation. Consistent with pro-proliferative effects in zebrafish, 5-ethyl-2′-deoxyuridine (EdU) labeling (10 μM, 1 hr) of whole hUCB cells exposed to 1,25(OH)D3 (1 μM, 4 hr) indicated a 35% increase ($p < 0.05$) in DNA synthesis in the CD34$^+$/CD45^{lo} HSPC population compared with controls (Figure 4B). 1,25(OH)D3-treated CD34$^+$ hUCB samples also showed significantly decreased cell death ($p < 0.05$), as determined by FACS for the apoptosis marker Annexin V (Figure 4C). To assess whether vitamin D3 would elicit a positive impact on in vitro expansion and function of mammalian HSPCs, CFU-C assays were performed. Ex vivo incubation of CD34$^+$ hUCB HSCs (n = 5 single donor hUCB units split for treatment and plated in replicate) with 1,25(OH)D3 for 4 hr at 37°C increased average total colony numbers at 7 days after plating by 38% ($p < 0.05$) compared with matched DMSO-treated controls (Figure 4D). This effect was concentration-dependent, where 1×10^{-7} and 1×10^{-6} M elicited a 39% ($p < 0.05$) and 56% elevation ($p < 0.01$) in colonies, respectively, whereas a higher dose of 1,25(OH)D3 (1×10^{-5} M) was refractory to expansion (Figure S4B). Analysis of the differentiation potential on day 10 after plating indicated that each progenitor fraction (colony forming unit, erythroid [CFU-E], colony forming unit, granulocyte/monocyte [CFU-GM], and colony forming unit, granulocyte/erythrocyte/monocyte/megakaryocyte [CFU-GEMM]) was increased by short-term, low-dose 1,25(OH)D3 exposure (1×10^{-6} M, $p < 0.0.05$) (Figure 4E). Further, the CFU-GEMM population in particular was significantly enhanced ($p < 0.05$), consistent with an impact on multipotent HSPCs.

Because VDR is a known transcriptional regulator, changes in gene expression due to 1,25(OH)D3 exposure relevant to the observed in vitro hematopoietic phenotypes were examined. Response to short-term 1,25(OH)D3 (4-hr) exposure was validated by qPCR, where, in addition to enhanced expression of *CYP24A1* (Figure 4A), the established VDR target and cell cycle regulator *G0S2* (Singh et al., 2014), and the anti-apoptotic *BCL2* (Vaux et al., 1988) were each elevated (Figure S4C). Further, although individual hUCB units showed differential responses to vitamin D3 stimulation, both Ingenuity Pathway (IPA) and MetaCore analyses revealed overlapping gene networks. In particular, IPA analysis indicated "IL-8 signaling" as a significantly regulated canonical pathway, whereas *IL-8 (CXCL8)* was a relevant component of three of the top ten MetaCore networks (Figure 4F; Figure S4D). The chemokine *CXCL8* was shown previously to contain a functional VDRE in its promoter (Ryynänen and Carlberg, 2013) and exhibit an early transcriptional response to 1,25(OH)D3 stimulation in HL60 cells (Savli et al., 2002). The CXCL8 receptors *CXCR1* and *CXCR2* are expressed in hUCB, including the $CD34^{+}$ fraction (Figure S4E). In response to 1,25(OH)D3 stimulation, $CD34^{+}$ hUCB HSCs showed a significant induction in *CXCL8* expression ($p < 0.05$) (Figure 4G). FACS analysis following monensin treatment to inhibit cytokine secretion confirmed a 40% increase ($p < 0.01$) in CXCL8 protein content in $CD34^{+}$ hUCB cells with 1,25(OH)D3 exposure (Figure 4H), indicating CXCL8 as a relevant VDR target in HSPCs.

1,25(OH)D3 Regulates CXCL8 Signaling to Modulate HSPC Production

We recently showed inflammatory activity stimulates developmental HSC production in zebrafish and mice (Li et al., 2014). CXCL8 is a potent regulator of chemotaxis, proliferation, and survival in the hemato-vascular system in response to inflammatory stimuli (de Oliveira et al., 2013). To investigate whether the effect of 1,25(OH)D3 on HSPCs was mediated by Cxcl8 signaling, modified epistasis studies were conducted in zebrafish. Embryos treated with 1,25(OH)D3 displayed elevated *cxcl8* expression, as assessed by WISH (Figure 5A) and quantified by whole-embryo qRT-PCR ($p < 0.05$) (Figure 5B). Moreover, qRT-PCR analysis of sorted cell fractions showed a 4-fold increase in *cxcl8* expression specifically in $Flk1^{+}cMyb^{+}$ HSPCs (Figure S5A). Consistent with autocrine or paracrine inflammatory stimulation of HSPCs, the Cxcl8 receptors *cxcr1* and *cxcr2* are expressed in the vascular and/or hematopoietic fractions of the embryo (Figure S5B). Further, injection of *cxcl8* mRNA elicited a dose-dependent expansion in HSPCs ($p < 0.05$) reminiscent of 1,25(OH)D3 treatment (Figure 5C). Use of a previously validated morpholino to *cxcl8* (Stoll et al., 2011) attenuated the shift in the fraction of embryos with elevated *runx/cmyb* expression (Figure 5D) as well as the boost in $Flk1^{+}cMyb^{+}$ HSPCs mediated by 1,25(OH)D3 (con versus 1,25(OH)D3, $p < 0.05$; con versus *cxcl8* MO, N.S.; *cxcl8* MO versus cxcl8 MO + 1,25(OH)D3, N.S.; 1,25(OH)D3 versus 1,25(OH)D3 + *cxcl8* MO, N.S.) (Figure 5E), resulting in no difference from controls. Likewise, co-treatment with the CXCR1/2 inhibitor SB225002 (White et al., 1998) reduced the overall response of HSPCs to 1,25(OH)D3 stimulation, as determined by WISH (Figure S5C) and FACS (Figure S5D) (con versus 1,25(OH)D3, $p < 0.01$; 1,25(OH)D3 versus SB25002, $p < 0.01$; con versus 1,25(OH)D3 + SB25002, N.S.; 1,25(OH)D3 versus 1,25(OH)D3 + SB25002, N.S.). Cxcl8-Cxcr1/2 signaling elicits functional responses via activation of a number of intracellular mediators. In particular, AKT signaling downstream of Cxcr1/2 stimulation influences apoptosis and proliferation (Waugh and Wilson, 2008). Exposure to 1,25(OH)D3 (12–36 hpf) increased total phosphorylated AKT (pAKT) levels by western analysis (Figure 5F). Significantly, this effect was not observed in *cxcl8* mutants. Likewise, in vitro exposure to the CXCL1/2 inhibitor SB225002 blocked KM HSPC expansion in the context of 1,25(OH)D3 stimulation in ZEST co-culture assays (Figure 5G) (con versus 1,25(OH)D3, $p < 0.0001$; con versus SB225002, $p < 0.05$; con versus 1,25(OH)D3 + SB25002, N.S.;1,25(OH)D3 versus 1,25(OH)D3 + SB25502, $p < 0.0001$). Finally, co-treatment with SB225002 antagonized functional enhancements in KM CFU numbers mediated by 1,25(OH)D3 or calcipotriol during in vitro methylcellulose culture (Figure 5H) (con versus 1,25(OH) D3, $p < 0.01$; con versus calcipotriol, $p < 0.01$; con versus SB225002, $p < 0.05$; con versus 1,25(OH)D3 + SB25502, N.S.; con versus calcipotriol + SB25502, N.S.). Together, our studies demonstrate that CXCL8-CXCR1/2 signaling functions downstream of 1,25(OH)D3-mediated VDR stimulation to directly regulate HSPC production and expansion.

DISCUSSION

Vitamin D has an established role in bone maintenance; severe developmental 1,25(OH)D3 deficiency results in abnormal bone mineralization (rickets) that can be treated clinically with vitamin D2 or D3 supplementation. Interestingly, VDR is expressed in many cell types, implying that it may have additional roles in other organs, including the hematopoietic system (Christakos et al., 2013; Plum and DeLuca, 2010). However, important differences in vitamin D biology and its regulatory impact on mammalian cells have been observed. In particular, transcriptional target genes relating to immune regulation are differentially regulated by vitamin D in mice compared with humans (Dimitrov and White, 2015). Further, vitamin D synthesis in mice is not exclusively dependent on Cyp2r1 as in humans, likely explaining the unexpected lack of phenotypes in knockout models (Zhu et al., 2013). In this study, we took advantage of the conservation of the vitamin D synthesis and signaling pathway in teleosts (Lin et al., 2012) and the amenability of zebrafish to chemical and genetic manipulation to characterize the direct impact of 1,25(OH) D3 availability on HSPC development. Here we found that active vitamin D3 is both necessary and sufficient to modify embryonic HSPC production. Further, we revealed that this effect is due to VDR-mediated transcriptional activation of the inflammatory chemokine *CXCL8*, stimulating cell-autonomous regulation of HSPC expansion and viability via CXCR1/2 activity.

Although CXCL8 is a potent chemo-attractant for neutrophils and functions as part of the inflammatory response (Deng et al., 2013), recent studies in zebrafish have indicated that it can modulate hematopoietic stem cell formation (Jing et al., 2015). Our findings support a model in which vitamin D/VDR-mediated induction of CXCL8 acts in an autocrine/paracrine manner to promote HSPC survival and proliferation, similar to its function in endometrial stroma cells (Arici et al., 1998). Prior reports indicate that, in contrast to BM and mobilized peripheral blood populations, CXCR1 and CXCR2 expression is highly enriched in hUCB HSCs (Rosu-Myles et al., 2000), perhaps indicating a fundamental difference in responsiveness to CXCL8 signaling between adult and gestational HSPC sources. Interestingly, although we observe an increase in CXCL8 expression in both zebrafish and hUCB in response to 1,25(OH)D3, the impact

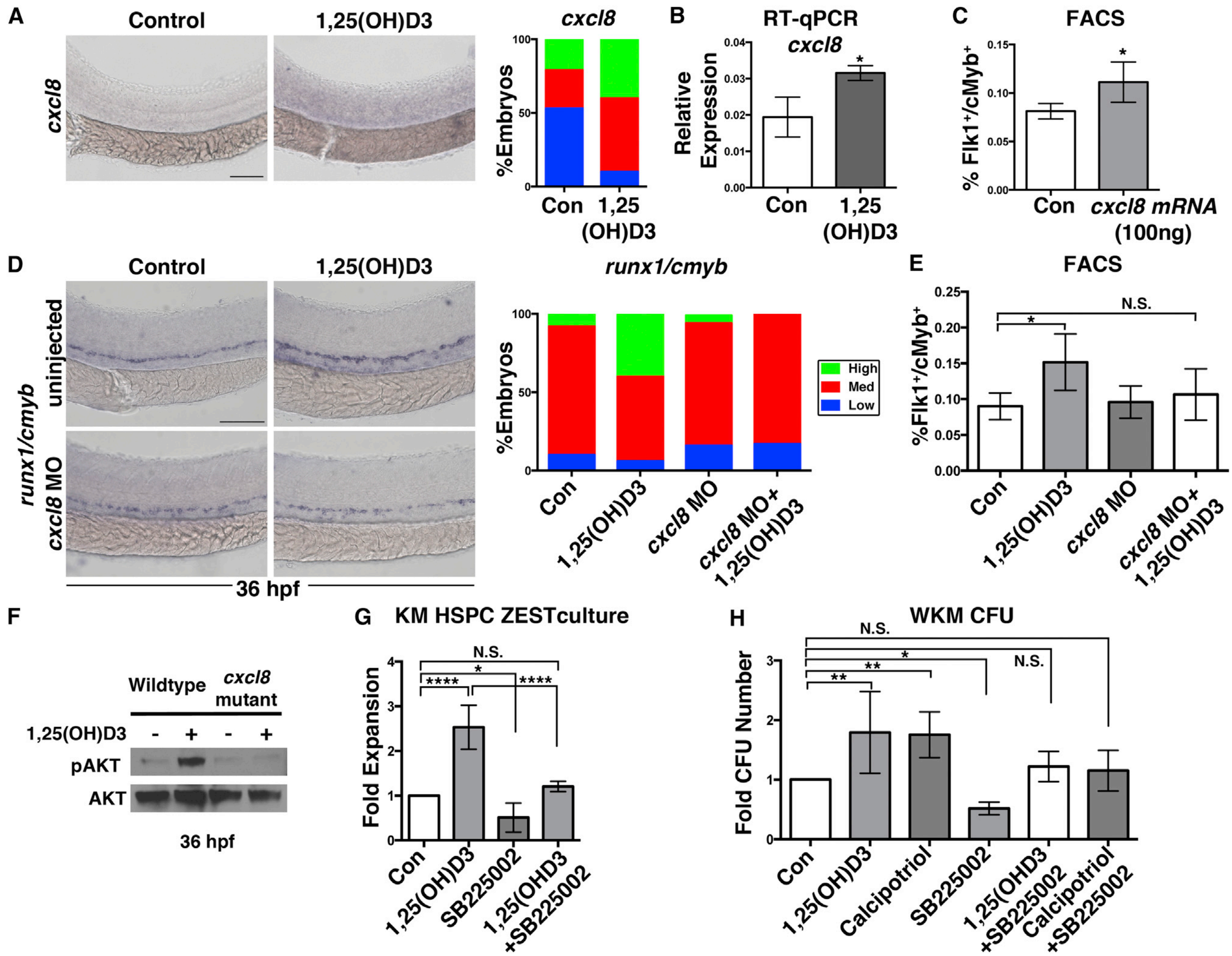

Figure 5. 1,25(OH)D3 Mediates HSPC Expansion through CXCL8 Signaling

(A) WISH for *cxcl8* showing elevated expression with 1,25(OH)D3 treatment at 36 hpf (n ≥ 30 embryos/condition).

(B) Whole-embryo qPCR confirmed increased *cxcl8* expression at 36 hpf after 1,25(OH)D3 exposure (*p < 0.05) (40 pooled embryos/condition × 3 replicates). Error bars, mean ± SD.

(C) FACS analysis of embryos injected with *cxcl8* mRNA (100 ng/embryo) showed increased Flk1:dsRed$^+$/cMyb:GFP$^+$ HSPCs at 36 hpf (*p < 0.05) (5 embryos/sample × 4 replicates/condition). Error bars, mean ± SD.

(D) WISH analysis and phenotype distribution indicated that the effect of 1,25(OH)D3 on *runx1/cmyb* expression was diminished in *cxcl8* morphants (n ≥ 35 embryos/condition).

(E) FACS analysis of MO-injected embryos treated with 1,25(OH)D3 (con versus 1,25(OH)D3, *p < 0.05; con versus *cxcl8* MO, N.S.; *cxcl8* MO versus cxcl8 MO + 1,25(OH)D3, N.S.; 1,25(OH)D3 versus 1,25(OH)D3 + *cxcl8* MO, N.S.)(5 embryos/sample × 4 replicates/condition; error bars, mean ± SD) showed that loss of *cxcl8* blocked vitamin D-mediated HSPC expansion.

(F) Western blot analysis for pAKT, with AKT as loading control, following 1,25(OH)D3 stimulation (12–36 hpf) in *cxcl8* mutants versus WT siblings (60 embryos/condition).

(G) WKM stromal cell assay documenting changes in proliferation by 1,25(OH)D3 with and without co-incubation with SB25002 (con versus 1,25(OH)D3, ****p < 0.0001; con versus SB225002, p < 0.05; con versus 1,25(OH)D3 + SB25002, N.S.; 1,25(OH)D3 versus 1,25(OH)D3 + SB25502, ****p < 0.0001) (n = 4; error bars, mean ± SD).

(H) WKM methylcellulose CFU assay showing fold increase with and without co-treatment of 1,25(OH)D3 with SB25002 (con versus 1,25(OH)D3, **p < 0.01; con versus calcipotriol, **p < 0.01; con versus SB225002, *p < 0.05; con versus 1,25(OH)D3 + SB25502, N.S.; con versus calcipotriol + SB25502, N.S.). n = 4 (SB25503 is n = 6); error bars, mean ± SD.

Scale bars, 100 μm. See also Figure S5.

of loss of Cxcl8-Cxcr1/2 signaling was most striking in the setting of vitamin D supplementation. These data imply that, although CXCL8 is a functional target of VDR stimulation, additional studies will be needed to identify whether other factors can contribute to overlapping or complementary aspects of VDR-mediated HSPC regulation in this or other contexts. Our findings also expand on the recent appreciation of the important role(s) of pro-inflammatory regulation in HSC induction and expansion during embryogenesis (Espín-Palazón et al., 2014; Li et al., 2014), which may serve as valid targets for therapeutic exploitation as proposed here.

Vitamin D has been investigated previously as a treatment for hematologic malignancy, albeit in a different context than presented here. Similar to use of retinoic acid for terminal differentiation of blasts in acute promyelocytic leukemia (APL), several in vitro studies showed that vitamin D treatment caused differentiation of leukemic precursors to the monocytic lineage (Hughes et al., 2010). These findings led to in vivo studies and phase 1 clinical trials for the use of vitamin D as a differentiation agent for acute myeloid leukemia (AML), which ultimately proved to be unsuccessful because of hypercalcemia and heterogeneity of the AML subtypes tested (Marchwicka et al., 2014). Interestingly, although treatment of the HL-60 leukemia cell line with 1,25(OH)D3 required 18–20 hr of exposure for differentiation to begin (Mangelsdorf et al., 1984), an initial burst in proliferation was seen during early phases (Brown et al., 1999), similar to the effect on HSPCs observed here. Our data indicate that 1,25(OH)D3 stimulates proliferative HSPC expansion. Consistent with this role, loss of 1,25(OH)D3 production or VDR function reduced HSPCs in vivo. Further, in vitro assays of both $CD41^+$ zebrafish HSCs and $CD34^+$ hUCB HSPCs revealed cell autonomous responses to short-term, low-dose 1,25(OH)D3 supplementation, independent of the niche, increasing multi-lineage CFU numbers via VDR-mediated alterations in proliferation and survival. These data are particularly relevant in the context of clinical observations of vitamin D-deficient pediatric and adult HSC transplant recipients who demonstrate a poor outcome, including graft versus host disease, delayed time to neutrophil engraftment, and overall survival (von Bahr et al., 2015; Wallace et al., 2015). Together, our data indicate that, beyond the published effects on lineage differentiation and immune-regulation, vitamin D has an important role in the foundation of the hematopoietic tree, regulating definitive HSPC numbers in the embryo.

Beyond the evolutionary conservation of 1,25(OH)D3/VDR-mediated regulation of HSPC numbers, cord-to-cord variability in our experimental and expression analyses highlight the potential clinical relevance of vitamin D status on hUCB function, particularly in regard to current ex vivo expansion and transplantation protocols. Vitamin D deficiency is a significant problem among pregnant women, ranging from 40%–50% in the northern United States (Bodnar et al., 2007). Extrapolating from these numbers, it is safe to assume that many hUCB samples are collected from vitamin D-deficient donors. In our hands, we observed variability in the magnitude of HSPC response among the treated hUCB samples, which we postulate may be attributed to the vitamin D status of the donor. Because ~50% of transplanted HSC units cause treatment-related complications, including graft failure (10%) and/or delayed time to transplant recovery (Singh et al., 2014), there are clearly still significant gaps in our understanding of the full array of factors contributing to donor HSPC function. In future investigations, it will be of scientific and clinical interest to obtain the vitamin D status of the donor (or sample) to determine whether the magnitude of in vitro expansion, transplant efficacy, and/or response to 1,25(OH)D3 stimulation depends on the original status of the unit. In sum, we have elucidated a fundamental role of vitamin D availability in regulating embryonic HSPC production, highlighting the functional potential of nutritional vitamin D supplementation for clinical hUCB expansion.

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry

Zebrafish lines were maintained and utilized in accordance with the Beth Israel Deaconess Medical Center (BIDMC) Institutional Animal Care and Use Committee (IACUC). The zebrafish lines used included AB, Tübingen (TU), and mutants and transgenics described in the Supplemental Experimental Procedures.

Chemical Treatments

Zebrafish embryos were exposed to chemical modulators in E3 fish medium from 12–36 hpf in multi-well plates unless noted otherwise. The following compounds (dose, supplier) were used: $1,25(OH)_2D3$ (1–-10 μM, Cayman Chemical), Calcipotriol (1–10 μM, Cayman Chemical), SB225002 (1 μM, Cayman Chemical), and 25(OH)D3 (10 μM, Tocris). Treated embryos were analyzed by WISH based on established protocols and utilizing published probes (Cortes et al., 2015). Phenotypic variation ($n \geq 20$ embryos/condition, $n \geq 3$ replicate clutches) was qualitatively analyzed as relatively high (up), medium (normal), or low (down) expression compared with age/stage-matched sibling controls and graphically depicted as the percentage falling into each of the three phenotypic expression bins. Normal expression reflected the most representative phenotype in the bell curve distribution of each cohort of control embryos per experiment. Images were acquired using a Zeiss Axio Imager A1/Axio Cam MRC using Axio vision LE software.

Morpholino and mRNA Injection

Translation blocking or splice-site MOs (Gene Tools) against ZF *vdra*, *vdrb*, *cyp27b1*, and *cxcl8* (Supplemental Experimental Procedures) were injected at the one-cell stage.

Embryo Dissociation and FACS Analysis

Fluorescent embryos (5 embryos/sample × 4 replicates/condition) were dissociated, resuspended in × PBS, and analyzed on a BD FACS Canto II in the presence of SYTOX Red dead cell stain (5 nM, Life Technologies) as described previously (Cortes et al., 2015). Data were analyzed using FlowJo X software (Tree Star). Double-negative, $Flk1^+/cMyb^-$, and $Flk1^+/cMyb^+$ cells were sorted (1,000 embryos/sample × 3 replicate sorts) on a BD SORP FACS Aria (BIDMC Flow Cytometry Core).

Expression Analysis

Total RNA was purified from zebrafish embryos (40 embryos/condition), human $CD34^+$ cells (500,000 cells/condition), or mononuclear UCB (one million cells/condition) using the RNAqueous Micro kit (Life Technologies) followed by Turbo DNase-I treatment (Life Technologies). For sorted populations, cDNA amplification was performed using the NuGEN Ovation Pico WTA2 system. For embryos, 1 μg of total RNA was used to generate cDNA using Superscript III First Strand Synthesis Supermix (Life Technologies). Superscript VILO mastermix was used for human RNA samples (<1 μg of RNA). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Life Technologies) on a Bio-Rad CFX384 Touch. Samples were run in triplicate with more than three biological replicates using published primers (Supplemental Experimental Procedures). Data analysis was performed using Real PCR Miner (http://ewindup.info/miner/).

Adult Kidney Marrow Analysis

KM from adult *cyp2r1* and *vdra* mutants and their wild-type (WT) siblings was dissected and analyzed by SSC versus FCS FACS profiling after red blood cell lysis as described previously (Stachura and Traver, 2016). Irradiation recovery was performed as described previously (North et al., 2007). Adult male zebrafish were exposed to 20 Gy of γ-irradiation and treated at 2 days post irradiation (dpi) with calcipotriol (20 μM) in the fish water. KM was dissected and analyzed at 10 dpi by FACS profiling on a Beckman Coulter Gallios flow cytometer (BIDMC Flow Cytometry Core). Percent HSPC recovery was analyzed by FlowJo X.

In Vitro Functional Analysis

Hematopoietic in vitro function assays were performed as described previously for zebrafish (Campbell et al., 2015; Stachura et al., 2011) and human cells (Goessling et al., 2011) and are detailed in the Supplemental Experimental Procedures.

Statistical Analysis

Statistical analyses were performed using Prism 6 (GraphPad). Two-tailed Student's t tests were used for pairwise comparisons and ANOVA for group analyses, with Holm-Sidak post hoc tests. Data are represented as mean ± SD. *$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$; N.D., not detected.

ACCESSION NUMBERS

The accession number for the microarray data reported in this paper is GEO: GSE86098.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.09.012.

AUTHOR CONTRIBUTIONS

M.C. designed all experiments. M.C. and S.Y.L. made mutant lines. M.C., M.J.C., and L.T.V. performed hUCB CFU assays. M.C., L.N.T., S.Y.L., I.M.F., and V.E. performed MO injections and WISH. M.C. and W.K. ran qPCR. M.C., D.L.S., and F.W. conducted zebrafish in vitro assays. T.M.S., G.Q.D., and W.G. provided reagents and guidance. T.E.N. and M.C. analyzed data and wrote the manuscript.

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