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HIF1α-induced PDGFRβ signaling promotes developmental HSC production via IL-6 activation

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Abstract

Hematopoietic stem cells (HSCs) have the ability to both self-renew and differentiate into all the mature blood cell lineages and thereby reconstitute the entire blood system. As such, HSCs are therapeutically valuable for treatment of hematological malignances and bone marrow failure. We recently showed that transient glucose elevation elicited dose-dependent effects on HSCs through elevated metabolic activity and subsequent ROS-mediated induction of Hypoxia Inducible Factor 1α (Hif1α). *Platelet Derived Growth Factor B (pdgfb)*, a Hif1α-target, and its receptor, *pdgfrb*, were significantly upregulated in response to metabolic stimulation. While the function of PDGF-signaling is well established in vascular development, its role in hematopoiesis is less understood. Exposure to either a pan-PDGF inhibitor or a PDGFRβ-selective antagonist in the context of Hif1α stimulation blocked elevations in HSPC formation as determined by *runx1;cmyb* WISH and HSPC-reporter FACS analysis. Similar results were observed for morpholino knockdown of *pdgfrb* or dominant negative *pdgfrb* expression, indicating PDGFRβ signaling is a key downstream mediator of Hif1α-mediated induction of HSPCs. Notably, overexpression of *pdgfb* ligand enhanced HSPC numbers in the AGM at 36hpf and in the CHT at 48hpf. A survey of known PDGF-B/PDGFRβ regulatory targets by qPCR revealed a significant increase in inflammatory intermediates, including interleukin 6 (IL-6) and its receptor (IL-6R). MO-mediated knockdown of *il6* or chemical inhibition of IL-6R antagonized the effect of *pdgfb* overexpression; furthermore, epistatic analysis of IL-6/IL-6R function confirmed activity downstream of Hif1α. Together these findings define a Hif1α-regulated signaling axis through PBFGB/PDGFRβ and IL-6/IL-6R that acts to control embryonic HSPC production.

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AUTHOR CONTRIBUTIONS

SL, VE, WK, IMF, LNT and SYL performed embryo exposures, MO injections, and *in situ* hybridizations. SL, MC and WK conducted FACS analysis and sorting. SL, VE and WK ran qPCR analysis and performed fluorescence microscopy. SL, VE and TEN designed experiments, evaluated results and wrote the manuscript. All authors reviewed and edited the manuscript. The authors have no conflicts of interest to declare.

INTRODUCTION

Hematopoietic stem cells (HSCs) possess the unique ability to both self-renew and differentiate into all the mature blood cell lineages and thereby reconstitute the entire blood system for a lifetime. In vertebrates, HSCs are initially generated during embryogenesis from specialized hemogenic endothelium in the ventral wall of the dorsal aorta, within an intraembryonic region termed the aorta-gonad-mesonephros (AGM) in mammals [1-4]. Runx1 is a transcription factor that is required for the production of definitive HSCs [5], controlling endothelial-to-hematopoietic transition (EHT) [6]. We previously determined that heightened glucose metabolism acts as an inductive trigger for production of $Runx1^{+}$ HSCs during embryogenesis through metabolism-mediated ROS production and subsequent stabilization of Hypoxia inducible factor 1α (Hif1α) levels *in vivo* [7].

The HIF complex functions as the master regulator of the adaptive response to low oxygen concentration, or hypoxia. When oxygen levels in the cellular environment are sufficient, Hif1α is targeted for degradation by the von Hippel-Lindau tumor suppressor protein (VHL). In contrast, hypoxic stimuli – including both pathological and relative decreases in oxygen content-inhibits Hif1α degradation, allowing it to interact with Hif1β to activate transcription of hypoxia responsive genes, which affect a wide range of cellular processes including glucose metabolism, cellular proliferation, erythropoiesis, and angiogenesis [8]. Our prior studies indicated Hif1α was necessary for HSC production in zebrafish embryos [7]. While complete loss of *Hif1a* is embryonic lethal prior to the formation of definitive HSCs in the mouse [9], more recent analysis using conditional loss of *Hif1a* function confirms it likewise regulates HSC development in the mammalian embryo [10]. Nonetheless, the factor(s) downstream of Hif1α-regulation that influence embryonic HSPC production are unknown.

Platelet Derived Growth Factors (PDGFs) belong to family of regulatory factors that control cell growth and proliferation. PDGFs act through protein tyrosine kinase receptors, PDGFRα and PDGFRβ. Binding of PDGFs to cognate receptors results in dimerization and activation of receptor tyrosine kinase activity, leading to the initiation of downstream cytoplasmic signal transduction pathways including the Phosphatidylinositol 3-kinase (PI3K), Extracellular Signal-regulated kinase (ERK), and Proto-oncogene tyrosine-protein kinase Src to affect migration, proliferation, and differentiation of PDGF responsive cell types [11]. Previous studies have shown that *PDGF-B* expression is regulated by hypoxia in cardiovascular and neuronal cell types as well as breast cancer cells [12-14]; *PDGF-B* was further documented to be a direct target of Hif1α in experiments using HeLa cells [14]. While PDGF-B has an established role in vascular remodeling during later stages of development and in adult angiogenesis via recruitment of PDGFRβ expressing pericytes [11], its mechanistic impact on hematopoiesis is less clear.

PDGF-B or *PDGFRb* knockout embryos die at birth of cardiovascular dysfunction and organ specific hemorrhages, exhibiting both anemia and thrombocytopenia, which are currently thought to be secondary to defects in other organs, including the heart, placenta, vasculature and liver [15, 16]. Previous *in vitro* and *in vivo* studies have indicated a role of PDGF-B in erythropoiesis [17-19] and megakaryopoiesis [20], but stimulation required the presence of

adherent stromal cells. PDGF has also been shown to act on platelets during wound healing, as well as stimulate pro-inflammatory cytokines from macrophages [21]. In mixed marrow cultures, incubation with recombinant PDGF-B enhanced colony formation of primitive hematopoietic precursors; however, it remains unclear whether PDGF directly affects hematopoietic function or indirectly stimulates HSPCs through the release of growth and differentiation factors from stromal cells [19]. Interestingly, irradiated wild-type mice can be reconstituted up to 4-12 months by grafting *PDGF-B* or *PDGFRb*-deficient hematopoietic cells [19], suggesting a non-cell autonomous function of PDGF-B signaling in adult HSC regulation. Finally, PDGF-B signaling in trophoblasts was more recently found to be a key component of the unique placental microenvironment that protects HSPCs from premature differentiation toward the erythroid lineage [22], indicating this growth factor pathway may exert additional hematopoietic regulatory impact during embryonic development.

PDGF/PDGFR signaling can stimulate the synthesis of various interleukins, including Interleukin-1 (IL-1β) and Interleukin 6 (IL-6), to affect cellular functions such as proliferation and migration in osteoblasts and smooth muscle cells [23]. IL-6 is a prominent pro-inflammatory cytokine produced during the immune response to infection [24] which acts by binding to the receptor complex made of the selective IL-6R (CD126) and the common signal transduction component gp130 (CD130). *In vitro*, IL-6, acting in synergy with IL-3, enhances the formation of multi-lineage blast cell colonies [25]. Consistent with these findings, *IL-6* deficient mice show a reduction in the number of primitive hematopoietic colony forming progenitor cells, as well as decreased long term reconstituting stem cell potential in transplantation assays [26]. IL-6 along with Flt3, SCF, and Thrombopoietin, have been shown to enhance the proliferation of primitive hematopoietic stem and progenitor cells *in vitro* [27, 28], making it an intriguing target for Hif1α-mediated regulation of developmental HSPC production.

Here, we demonstrate a role for PDGF-B/PDGFRβ signaling downstream of Hif1α activation in the regulation of HSPC production during embryonic hematopoietic development. Inhibition of PDGFRβ signaling in the presence of chemical or genetic Hif1α stabilization attenuated its ability to enhance HSPC formation. In contrast, overexpression of *pdgfb* robustly increased HSPC production, even in the absence of HIF1α function. The effect of PDGF-B/PDGFRβ was mediated by IL-6/IL-6R activity, whereby loss of the ligand or receptor antagonism could block PDGF-induced HSPC expansion. Finally, IL-6 was both induced by and determined to function downstream of Hif1α stabilization. Together these findings uncover a signaling axis through PDGF-B/PDGFRβ and IL6/IL6R that regulates the scale of definitive HSPC formation via inflammatory signals in response to Hif1α stimulation.

METHODS

Zebrafish husbandry

Zebrafish were maintained according to Beth Israel Deaconess Medical Center IACUC-approved protocols. Tg(−6.0itga2b (CD41):eGFP [29], Tg(kdrl (flk1):dsRed), *Tg(cmyb:eGFP), Tg(gata1:dsred)* and *Tg(EPV.Tp1CMmu.Hbb (Notch reporter):EGFP)*

lines were described previously [30, 31]. The *pdgfrb* mutant line was previously published [32]. The *hsp70:ca-pdgfrb* and *hsp70:dn-pdgfrb* lines were created as detailed below.

Chemical treatments and evaluation

Zebrafish embryos were exposed to compound-modifiers in E3 water in multi-well plates for durations noted. Compounds utilized were: DMPQ (10μM, Cayman), cobalt (II) chloride ($CoCl_2$, 500μM, R&D Systems), DMOG (75μM, Cayman), AG1295 (10μM, Calbiochem), AG1296 (2.5μM, Cayman), and SC-144 (1μM, EMD Millipore). Whole-mount *in situ* hybridization (WISH) was performed as previously described [33]. Qualitative phenotypes for individual embryos (n≥20 embryos/condition, ≥2 replicate clutches) were scored as relatively high/medium/low in expression compared to sibling controls and graphically depicted as the percentage falling into each of the 3 phenotypic expression bins; “medium” expression was set as the most representative phenotype in the normal bell-curve distribution of each cohort of control embryos per experiment.

Fluorescence Activated Cell Sorting

FACS analysis was performed using double transgenic *Tg(flk1:dsRed;cmyb:GFP)* or *Tg(CD41:GFP;gata1:dsRed)* reporter embryos as previously described [1]. Embryos (pools of 3-5 embryos per sample, ≥5 replicates) were dissociated, resuspended in 1×PBS, and analyzed on a BD FACSCanto II (BD Biosciences, San Jose, CA) in the presence of SYTOX Red Dead Cell Stain (5nM, Life Technologies, Waltham, MA). Data was analyzed using FlowJo X software (TreeStar, Ashland, OR). For isolation of endothelial (Flk1:dsRed$^+$cMyb:GFP$^-$) and HSPC (Flk1:dsRed$^+$cMyb:GFP$^+$) fractions, pooled embryos (n>1000) were sorted using FACSAria (BD Biosciences, San Jose, CA). After cell collection, RNA was extracted, treated with DNaseI (RNAqueous-Micro Total RNA isolation Kit, Life Technologies, Rockville, MD), and amplified with Ovation RNA Amplification System V2 (NuGEN).

Morpholino and mRNA Injection

Morpholinos to *vhl* and *hif1a* (GeneTools, Philomath, OR) were injected as described previously [7, 34]; MO sequences are listed in the **Supplemental Methods**. Briefly, each MO (dose: 0.2-0.4mM) was microinjected into 1-cell stage embryos and allowed to develop to the timepoint(s) of interest before processing with matched sibling controls for evaluation as above. For mRNA generation, *pdgfb* and *il6* Coding Data Sequences were amplified by PCR (*see primers pairs below*) from IMAGE clones 6330609 and 40130735 respectively and cloned into pCS2+ (EcoRI/XbaI). mRNA was *in vitro* transcribed from NotI linearized constructs using the SP6 mMESSAGE mMACHINE kit (Life Technologies) and injected at the 1-cell stage. Mouse *Pdgfb* mRNA was injected at 25ng/μl, mouse *Il6* mRNA was injected at 200ng/μl, and *dnhif1* mRNA [35] was injected at 200ng/μl.

Pdgfb-

F: 5’ GATGGAATTCATGAATCGCTGCTGGGCG

R: 5’ GATGTCTAGACTAGGCTCCGAGGGTCTCC

Il6-

F: 5’ GATGGAATTCATGAAGTTCCTCTCTGCAAG

R: 5’ GATGTCTAGACTAGGTTTGCCGAGTAGATC

Generation and induction of the hsp70:ca-pdgfrb and hsp70:dn-pdgfrb expression constructs

Mouse *pdgfrb* Coding Data Sequence was amplified from the IMAGE clone 30060666 and cloned into the pENTR-D-TOPO vector (Invitrogen) using the following primers:

F 5’ CACCATGGGGCTTCCAGGAGTGATACCAG

R 5’ CTACAGGAAGCTGTCCTCTGCTTCAGCC

The D849V amino acid substitution used to generate the constitutively activating mutation (*ca-pdgfrb*), as described in murine *Pdgfrb* [36], was created by site-directed mutagenesis. The *dn-pdgfrb* construct, which possesses a truncation of the receptor tyrosine kinase domain, was generated by PCR amplification with the following primers and cloned into the pENTR-D-TOPO vector:

F 5’ CACCATGAAGAGTTCGACCATCAG

R 5’ CTATCTTCCTCCACACAGCAATG

The *hsp70:ca-pdgfrb* and *hsp70:dn-pdgfrb* constructs were generated using the multigateway LR Clonase (Invitrogen) Gateway reaction. The resulting plasmid was microinjected with *tol2*-transposase RNA into 1-cell-stage embryos [37]. Tg(*hsp70:ca-pdgfrb)* and WT sibling control embryos were heat-shocked at 27hpf by incubation in a 37°C water bath for 1 hour. *Tg(hsp70:dn-pdgfrb)* and WT sibling controls were heat shocked with the same conditions, and split for additional $CoCl_2$ treatment following the incubation. Embryos were fixed at 48hpf for analysis and processed as above.

Microscopy

Fluorescent embryos were treated as above and imaged by fluorescence microscopy using a Zeiss Discovery V8/Axio Cam MRC and Axiovision LE software (Carl Zeiss), as previously described [34]. Cell counts were quantified using ImageJ (NIH). Two-tailed Student's *t*-tests were performed for investigational comparisons; one-tailed tests were run for statistical confirmation of WISH phenotypes. Data are presented as mean ± SEM, and p-values < 0.05 were considered significant.

Quantitative RT-PCR

qPCR was performed on cDNA isolated from pooled embryos at timepoints indicated (n=25 embryos/variable; see **Supplemental Methods** for qPCR primers) using ABI PRISM 7900HT (Invitrogen). Samples were run in technical triplicate with ≥3 biological replicates/condition. Ct values were determined using PCR Miner [38] and fold-change calculated by the ddCt or R0 method with *tbp* or *B-actin* as the reference gene.

Statistical Analyses

Two-tailed Student's *t*-tests were performed for statistical analysis and data presented, unless otherwise indicated. For confirmation of WISH results, one-tailed Student's t-tests were performed based on predicted outcome.

RESULTS

Hif1α stabilization enhances HSPC production via up-regulation of PDGF-B signaling

We previously identified *platelet-derived growth factor* (*pdgf*) as a potential target of Hif1α relevant to embryonic HSPC production by gene expression analysis [7]. To assess whether PDGF signaling functions downstream of Hif1α in developmental HSPC production, we first conducted modified epistasis experiments: zebrafish embryos were exposed to the pan-PDGF receptor inhibitor, AG1295 (10μM), in the presence or absence of the known Hif1α agonist $CoCl_2$ (500μM) during the period of HSPC specification from 12-36hpf. Embryos treated with $CoCl_2$ exhibited qualitatively stronger expression of *runx1*;*cmyb* in the ventral dorsal aorta (VDA) compared to age-match sibling controls as visualized by whole mount *in situ* hybridization (WISH), consistent with our prior analysis [7, 33] **(Fig 1A,B)**. In contrast, the majority of embryos exposed to AG1295 displayed reduced *runx1*;*cmyb* expression in the VDA; furthermore, AG1295 blocked the $CoCl_2$-mediated increase in the distribution of embryos with enhanced HSPC gene expression **(Fig 1A,B)**. This effect was confirmed and quantified by flow cytometry using the *Tg(−6.0itga2b(CD41):eGFP* line (crossed to *Tg(gata1:dsRed)* to exclude thrombocytes), which has been previously shown to mark HSPCs with *in vivo* repopulating potential [7]. Exposure to AG1295 caused a 0.58-fold reduction ($p<0.001$) in $CD41^+$;$Gata^-$ HSPCs (**Fig 1C**), and blocked the ability of $CoCl_2$ to enhance HSPC numbers ($p<0.05$), suggesting that Hif1α influences HSPC development in part through PDGFR signaling.

To determine which PDGF family members are the primary targets of Hif1α in this context, embryos were exposed to $CoCl_2$ during HSPC specification and PDGF-related gene expression was examined by whole embryo RT-qPCR. $CoCl_2$ treatment significantly increased gene expression of *pdgfba* ($p<0.05$; the gene encoding the PDGFb ligand is duplicated in zebrafish: *pdgfba* and *pdgfbb*) and *pdgfrb* ($p<0.05$); notably, expression of related factors were not statistically altered (**Fig 1D**). As confirmation of regulatory specificity, embryos were either injected with a previously published MO targeting Von Hippel-Lindau (VHL), an E3 ubiquitin ligase that normally targets hydroxylated Hif1α to the proteasome for degradation when oxygenation is sufficient, or exposed to DMOG (75μM), a prolyl hydroxylase inhibitor that leads to Hif1α stabilization and increased *runx1* expression [7]. Each treatment resulted in increased expression of *pdgfba* ($p<0.01$) and *pdgfrb* ($p<0.05$) by RT-qPCR **(Fig 1E** and **Fig S1A)**, but no significant differences in closely related family members. WISH analysis indicated that *pdgfrb* is expressed in the trunk region encompassing the VDA at 36hpf, consistent with prior observations [39]; furthermore, increased local expression of *pdgfrb* was documented in embryos exposed to $CoCl_2$ (**Fig 1F,G**). RT-qPCR analysis of sorted cell populations from Tg(*flk1:dsRed*;*cmyb:GFP*) embryos at 36hpf confirmed that *pdgfba* and *pdgfrb* are expressed in both $Flk1^+$;$cMyb^-$ endothelium and $Flk1^+$;$cMyb^+$ HSPCs (**Fig S1B**), with each

also found in the negative fraction. To identify which cell types may be responding to Hif1α regulation through PDGFRβ signaling, we compared expression levels of *pdgfba* and *pdgfrb* in sorted cell populations from *flk1:dsRed;cmyb:GFP* embryos that had been treated with $CoCl_2$ (12-36hpf) to matched DMSO controls. As expected, the established Hif1α target *erythropoietin receptor* (*epor*) was robustly induced following treatment. RT-qPCR analysis showed that expression of *pdgfrb* was increased in all sorted fractions (**Fig S1C**); interestingly, *pdgfba* expression was increased specifically in the $Flk^{+}cMyb^{-}$ endothelial population, similar to that seen for *runx1,* implying PDGF activity may directly impact hematovascular commitment and/or subsequent HSPC production downstream of Hif1α stimulation [7]. As PDGF signaling impacts vascular remodeling [40], we assessed the effect of AG1295 treatment, as well as a targeted PDGFRβ-selective inhibitor, DMPQ (10μM), on Notch-dependent hemogenic specification. No differences were observed in *flk1* (endothelial) or *ephrinb2a* (arterial) expression at 36hpf at the doses utilized (**Fig S1D,E**); in contrast, while AG1295 dramatically reduced gfp expression in the VDA using the Notch reporter line, *Tg(EPV.Tp1CMmu.Hbb:EGFP)* [31], DMPQ had no effect on hematovascular commitment (**Fig S1F,G**). Together, these data suggest that Hif1α stabilization can enhance HSPC production, independent of hemogenic specification, via the up-regulation of *pdgfba* and *pdgfrb* expression

PDGFRβ signaling is required downstream of Hif1α to control AGM HSPC production

To determine if PDGFRβ activity was necessary for HPSC formation in the VDA, we employed a gene knockdown approach using a previously validated *pdgfrb* morpholino [41]; embryos injected with *pdgfrb* MO had no gross aortic phenotype at 36hpf at the doses utilized as determined by *flk1* expression, consistent with DMPQ exposure (**Fig S1D**) and prior reports [32](**Fig S2A**). In contrast, *runx1;cmyb* staining was reduced but not eliminated in half of the embryos examined (**Fig S2B,C**). Similar findings were observed using a recently described *pdgfrb* mutant [32]; however, while homozygous embryos did not exhibit a strong HSPC phenotype, the majority of heterozygous siblings showed reduced *runx1;cmyb* expression in the VDA (**Fig S2D,E**). This data suggests potential compensatory interactions are elicited by complete *pdgfrb* loss (perhaps by the closely related *pdgfra*) and highlights that dosage-dependent PDGFRβ regulation alone is active in, but not essential for, developmental HSPC specification.

As Hif1α stimulation specifically stimulated *pdgfrb* expression, we next sought to determine if PDGFRβ signaling was required to mediate its effect on HSPC numbers using modified epistasis analysis. Stabilization of Hif1α via DMOG treatment increased *runx1;cmyb* staining in control embryos, consistent with our prior analysis [7]; in contrast, DMOG was unable to elicit elevated expression in *pdgfrb* morphants (**Fig 2A,B**). Similar observations were seen for chemical inhibition of PDGFRb using DMPQ in the setting of Hif1a stimulation (**Fig S2F,G**). The requirement of PDGFRβ activity downstream of Hif1α was further validated using the *vhl* MO. Consistent with stabilized Hif1α levels in the absence of VHL function and our prior observations [7], *vhl* morphant embryos had enhanced *runx1*;*cmyb* expression in the VDA; however, this effect was also not observed in *pdgfrb* morphants (**Fig 2C,D**). To confirm and quantify these findings, FACS for CD41:GFP was performed at 36hpf, and demonstrated that the significant increase in HSPCs caused by loss

of VHL-mediated Hif1α regulation ($p<0.05$) was blocked by *pdgfrb* knockdown ($p<0.05$) (**Fig 2E**). Finally, to ensure the effects on HSPC numbers were not due to embryonic toxicity impacting aortic specification and/or function, and to further confirm the specificity of the role of *pdgfrb*, a dominant negative construct was employed. Heat shock induction at 28hpf, after hemogenic endothelial specification and the onset of circulation, had modest impact alone, yet dramatically blocked the impact of $CoCl_2$ stimulation by WISH at 48hpf (**Fig 2F,G**). This effect was confirmed by $CD41^+$ HSPC cell counts, whereby expression of *dn-pdgfrb* significantly blocked the ability of $CoCl_2$-mediated Hif1α activation to increase HSPC numbers (**Fig 2H**). Together, these findings indicate that PDGFRβ is a functionally relevant target of Hif1α in embryonic HSPC regulation.

pdgfb overexpression increases HSPC production in the absence of Hif1α

To next determine if PDGF signaling plays an instructive role in embryonic HSPC production in the AGM, we overexpressed murine *pdgfb* mRNA in the zebrafish embryo. Embryos injected with 25ng/μL *pdgfb* mRNA exhibited enhanced *runx1*;*cmyb* expression (**Fig 3A,B**) and increased $Flk1^+$;$cMyb^+$ HSPC counts in the AGM at 36hpf (Control: 6±1.5 cells/AGM, *pdgfb* mRNA: 10.4±2.2, n≥15/condition, $p<0.0001$) (**Fig S3A,B**). Furthermore, this effect was sustained at 48hpf as assessed by *cmyb* expression in the CHT (**Fig S3C,D**) and quantified by $Flk1^+$;$cMyb^+$ flow cytometry (1.54-fold increase vs. control, $p<0.0001$) (**Fig 3C**). Analysis of embryos expressing *Tg(−6.0itga2b(CD41):eGFP* confirmed a significant increase in CD41:GFP^+ HSPCs in the CHT following overexpression of murine *pdgfb* (Control: 13.1±7.4 cells/CHT, *pdgfb* mRNA: 18.9±9.8, n≥35/condition, $p<0.0053$) (**Fig S3E,F**). As PDGF-B could stimulate multiple PDGFRs, a heat shock inducible construct was created to investigate the effect of specific upregulation of PDGFRβ activity. Induction of constitutively active *pdgfrb* at 27hpf mimicked the effects of *pdgfb* overexpression, enhancing *cmyb* expression at 48hpf (**Fig 3D,E**) and increasing the total number of $Flk1^+cMyb^+$ HSPCs as determined by FACS analysis ($p<0.05$) (**Fig 3F**). Finally, to assess whether increased PDGF-B/PDGFRβ signaling could rescue the effect of reduced Hif1α activity on HSPC production in the VDA a modified epistasis experiment was conducted. Consistent with our prior observations [7], *runx1*;*cmyb* expression was reduced in *hif1α* morphants. Overexpression of *pdgfb* mRNA in *hif1α* morphant embryos enhanced the proportion of embryos showing elevated *runx1*;*cmyb* levels (**Fig 3G,H**). Together these data indicate that PDGF-B/PDGFRβ receptor stimulation is functionally conserved across vertebrate species and is sufficient to increase HSC induction from the hemogenic endothelium.

IL-6 is required downstream of PDGFRβ to stimulate AGM HSPC expansion

To understand the mechanism by which PDGF-B/PDGFRβ signaling regulates HSPC formation, we examined previously identified PDGF-B regulatory targets known to impact vascular and/or hematopoietic cell types [42]. Expression of inflammatory mediators *il1b*, *il6*, *mmp2* and *mmp9* were increased in 36hpf embryos injected with *pdgfb* mRNA, with *il6* was the most strongly up-regulated (4.6-fold change; $p<0.05$) (**Fig 4A** and **Fig S4A**). In addition, *pdgfb* overexpression also significantly increased expression of the *il6 receptor (il6r)* and its co-receptor, *gp130* (*il6st*) (**Fig 4A**), suggesting that IL-6 signaling could be a

functionally relevant target of PDGF-B/PDGFRβ in regulation of embryonic HSPC production. RT-qPCR analysis of sorted cell fractions of wild-type embryos at 36hpf showed that *il6r* and *gp130* are relatively enriched in $Flk1^{+};cMyb^{-}$ endothelium and $Flk1^{+};cMyb^{+}$ HSCs **(Fig S4B)** at this stage of development. Modified epistasis analysis demonstrated that MO-mediated knockdown of *il6* only modestly impacted *runx1* expression in the VDA at 36hpf (**Fig 4B,C**), consistent with its low baseline level of expression (**Fig S4B**). However, *pdgfb* overexpression could no longer increase *runx1*;*cmyb* expression in the AGM in the absence of IL-6 function mediated either by MO knockdown (**Fig 4B,C)** or chemical inhibition of IL-6R/gp130 with the chemical antagonist SC-144 (**Fig S4C,D**). These findings were confirmed and quantified by $Flk1^{+};cMyb^{+}$ cell counts in the VDA at 36hpf (Control: 2.9±1.2 cells/AGM, *pdgfb* mRNA: 5.1±1.7, *il6* MO: 3.5±1.7, *pdgfb* mRNA+*il6* MO: 3.6±1.9, n≥15/condition, *p<0.05, ***p<0.0005) (**Fig 4D**) and together indicate IL-6 activity is a functionally relevant mediator of PDGFRB stimulation on HSPC development. In support of that conclusion, overexpression of *il6* mRNA increased *runx1* expression in the AGM at 36hpf (**Fig S4E,F**) as well as the percentage of $Flk1^{+};cMyb^{+}$ HSPCs at 48hpf as assessed by FACS (1.49-fold vs. control, p≤0.02) (**Fig 4E**). Furthermore, *il6* mRNA significantly elevated the number of phospho-histone H3 (pH3)-positive cells in the VDA region, similar to that seen with *pdgfb* injection (Control: 5.1±2.5 cells/AGM, *pdgfb* mRNA: 7.9±2.5, p≤0.014; *il6* mRNA: 7.4±2.2, p≤0.002, n≥10/condition) (**Fig 4F**). Altogether, these data indicate that IL-6 functions downstream of PDGF-B/PDGFRβ to increase HSPC production in the developing embryo via proliferative expansion.

IL-6 signaling acts downstream of Hif1α to increase AGM HSPC production

Our previous studies indicated that elevated glucose metabolism caused ROS-mediated Hif1α stabilization, leading to increased developmental HSPC specification and proliferative expansion [7]. To further strengthen the regulatory network connecting Hif1α and PDGFRβ signaling to HSC regulation via IL-6, we directly examined the role of IL-6 signaling downstream of Hif1α induction on embryonic HSPC formation. Hif1α stabilization with $CoCl_2$ (12-36hpf) significantly up-regulated the expression of *il6* (p<0.05) and its receptor *il6r* (p<0.01) by RT-qPCR (**Fig 5A**); furthermore, analysis of sorted HSPC fractions showed the predominant responses were found in the $Flk1^{+};cMyb^{-}$ endothelial and $Flk1^{+};cMyb^{+}$ HSC populations (**Fig S5A**), similar to that seen for *pdgfb* and *pdgfrb*. Modified epistasis experiments indicated that blocking IL-6 signaling using either the *il6* MO or SC-144 partially suppressed the effect of $CoCl_2$ on *runx1*;*cmyb* expression in the AGM (**Fig 5B,C** and **S5B,C**). This finding was confirmed and quantified by $CD41^{+}$ HSPC analysis using FACS: whereas $CoCl_2$ normally induced HSPC numbers, this effect was absent in the context of *il6* knockdown (**Fig 5D**), suggesting that IL-6 signaling acts to further mediate the effect of Hif1α on embryonic HSPC number. Finally, to determine whether IL-6 signaling is sufficient to increase HSPC induction in the absence of Hif1α activity, similarly to that observed for PDGF-B, embryos were co-injected with *dominant-negative hif1a* (*dnhif1a*) [35] and *il6* mRNA. Inhibition of Hif1α function decreased *runx1*;*cmyb* expression in the AGM, consistent with prior analysis [7]; importantly, overexpression of *il6* mRNA was able to restore *runx1*;*cmyb* expressing HSPCs to levels seen in controls (**Fig 5E,F**), indicating that IL-6 signaling functions downstream of Hif1α to influence HSPC production. Collectively, our data reveal a PDGF-associated signaling network connecting Hif1α-

stabilization to IL-6 signaling that provides a method to regulate HSPC production through inflammatory cascades in response to environmental stimuli during development.

DISCUSSION

The generation and production of HSCs from hemogenic endothelium is an intricate and multifaceted process involving multiple signaling mechanisms and molecular inputs that are spatially as well as temporally regulated [43, 44]. It is becoming increasingly clear that environmental and external factors can act upon and feed into the normal process of HSC generation, given that the developing embryo is exposed to fluctuating levels of oxygen, nutrients and energy supply [7, 45]. The importance of the hypoxic sensor Hif1α is now well documented in the adult BM niche, where local hypoxia and resultant Hif1α levels serve a key physiological mechanism to regulate HSC number and intracellular damage by maintaining low metabolic rate and HSC cell cycle quiescence [46]. Interestingly, we previously showed that Hif1α can also regulate embryonic HSC numbers and function, in this case by mediating the metabolic response to glucose metabolism to control induction of HSCs from the hemogenic endothelium and their subsequent proliferative expansion in the embryo [7]; a role for Hif1α as an inductive rather than quiescent factor was similarly demonstrated in mammalian embryos [10]. While Hif1α has been long established to act as a physiological sensor to regulate downstream targets such as Erythropoietin (Epo) and Vascular Endothelial Growth Factor (VEGF) to maintain erythroid homeostasis and vascular remodeling in response to hypoxia [47], the functional targets of Hif1α that could act to stimulate HSPC production in an expansive niche remain to be identified. In this paper, we show a novel mechanism of action mediated by Hif1α, which through regulation of PDGFRβ signaling, ensures proper production of HSCs through regulation of proproliferative inflammatory signaling during periods of metabolic stimulation throughout embryonic development.

Expression of PDGF-B and its receptor is induced by glucose exposure and the subsequent increase in metabolic activity, including ROS production, [7] as well as direct chemical and/or genetic-mediated Hif1α stabilization. PDGF-B has previously been shown to promote the ability of primitive hematopoietic precursor cells to form multilineage colonies in culture, as well as the *ex vivo* expansion of $CD34^+$ human cord blood cells, although it has been unclear whether the expansion is a result of direct stimulation or secondary to autologous effects on stromal supporting cells [21, 48]. Our results show that PDGF-B signaling is necessary for the Hif1α-mediated induction of HSCs, and that overexpression of PDGF-B is sufficient to stimulate increased HSPC production during hematopoietic development *in vivo*. Recently, PDGF-B activity in trophoblasts was found to be a vital component of the placental niche where it functions to protect HSPCs from premature differentiation into red blood cells by suppressing the production of EPO [22]; this data together with our own presented here suggests that PDGFB may be playing a role in regulating the balance between HSPC maintenance and differentiation during development. In our studies, we further identify IL-6 signaling downstream of PDGFRβ activity in mediating the effect of metabolic induction of HSCs, as the effect of PDGF-B overexpression on HSPCs number can be attenuated by morpholino knockdown of IL-6. Inflammatory cytokines have been well characterized to activate the mobilization,

proliferation, and differentiation of HSCs to ensure an adequate response to infection or injury during demand-driven hematopoiesis [49, 50], and have also recently been shown to play an important role in embryonic HSC development [51-54]. We have identified IL-6 as an additional inflammatory factor that can influence magnitude of developmental HSC production under the control of the Hif1α-PDGFRβ signaling axis. Interestingly, hypoxia has been shown previously to induce the expression of cytokines and proinflammatory mediators, including IL-6 [55, 56]. Interestingly, studies involving murine marrow cells cultured ex vivo had documented that IL-6 in combination with physiologically low oxygen concentration (1%) improved the maintenance of primitive HSC subpopulations [57] and more recent studies have reported that addition of IL-6 to hypoxia mimicking culture conditions can enhance in vivo long term reconstituting hematopoietic stem cell potential [58]. As there exist many regulatory inputs that converge to influence hematopoiesis, IL-6 may serve as a regulatory node where key environmental stimulus signal into to ensure right number of HSC production and balance of hematopoiesis. In our studies, *pdgfb* expression was found to be increased specifically in the endothelial cells in response to $CoCl_2$ mediated Hif1α stabilization, while *pdgfrb* was found upregulated in both the endothelial as well as hemogenic endothelial/HSC population. IL-6 and IL-6R were similarly upregulated in both the endothelium and HSPCs in response to $CoCl_2$. While significant further investigation involving cell-type specific studies is warranted, we propose a possible mechanism of action whereby PDGF-B, acting through both an autocrine and paracrine mechanism, can influence IL-6/IL-6R signaling to thereby regulate the overall production of HSPCs.

While mechanistic investigations into how growth factor signaling involving PDGF-B can lead to the activation of IL-6 were beyond the scope of this study, there are many other reports that provide insight into the myriad of possible routes of regulation. In osteoblastic cells, Protein Kinase C (PKC) activation of members of the activator protein-1 (AP-1) complex was determined as having a direct role in the PDGF-B induction of IL-6 [23]. In contrast in glioma-initiating cells, a PDGF-driven signaling axis involving NO-dependent inhibitor of differentiation 4 (ID4) has been shown to promote JAGGED1–NOTCH activity [59]. Related, both direct and non-canonical regulation of IL-6 expression by Notch has been previously reported [60, 61]. As it is well established that generation of HSCs requires multiple Notch signaling inputs [62], it will be interesting to investigate whether Notch signaling may be a part of the Hif1α-PDGFRβ signaling axis in regard to the ability of IL-6 to enhance production of HSPCs. Furthermore, while we report increased proliferation as assessed by $pHH3^+$ cell counts following *pdgfb* and *il6* overexpression, whether we are affecting hemogenic endothelial competence for HSC generation or the proliferation of specified HSCs is complicated by the fact that both functions are contemporaneous. Future studies involving live cell imaging using transgenic reporters labeling endothelial and hematopoietic populations may help clarify the exact process in HSPC development that is altered by the Hif1α-PDGFRβ-IL-6 signaling axis.

In summary, we have shown that during development, PDGFRβ can function downstream of Hif1α signaling to regulate the scale of definitive HSPC formation via IL-6 mediated inflammatory regulation. We anticipate that further understanding of exactly how environmental and physiological inputs can induce cell signaling events, in this case metabolic induction of PDGFRβ signaling, to affect the normal course of HSPC production

in vivo will provide new insights into mechanisms to improve *in vitro* generation or expansion of HSCs for research and therapeutic purposes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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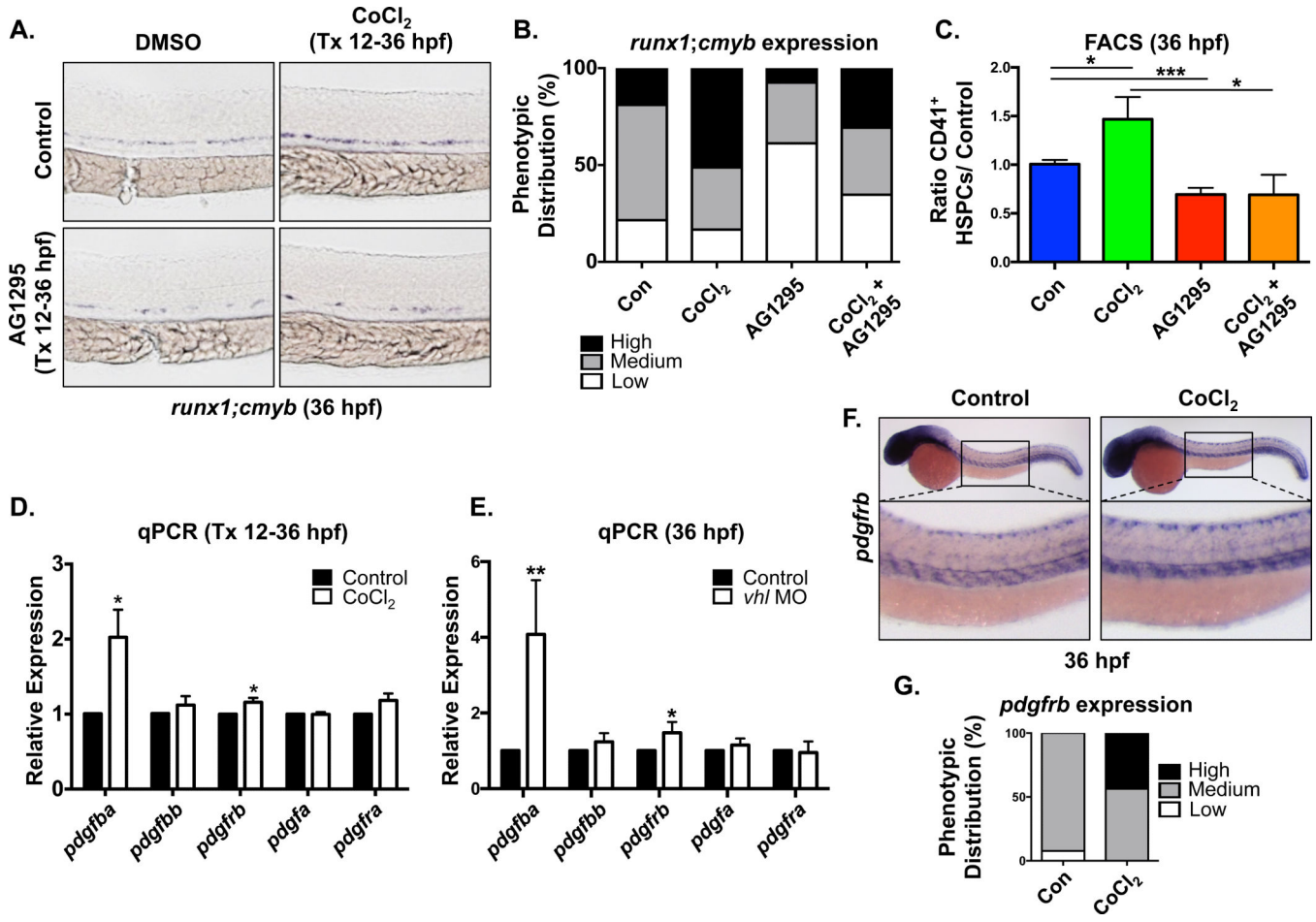

Figure 1. Hif1α stabilization enhances HSPC production via PDGF activity

(A) Embryonic exposure to the pan-PDGFR inhibitor AG1295 (10μM) (during HSC formation (12-36hpf) decreased *runx1;cmyb* WISH expression in the AGM and blocked the increase in *runx1;cmyb* expression due to exposure to Hif1α agonist ($CoCl_2$, 500μM). Tx = treatment.

(B) Qualitative phenotypic distribution of embryos from panel 1A scored with low, medium or high *runx1;cmyb* expression in the AGM (n≥20 condition × 3 replicate clutches).

(C) FACS analysis confirmed that chemical stabilization of Hif1α enhanced HSCs, while inhibition of PDGFR decreased $CD41^{+}gata1^{-}$ HSPCs at 36hpf in the presence of $CoCl_2$ (*p<0.05, ***p<0.001, one-tailed t-test, n≥13 replicates/condition).

(D) RT-qPCR analysis showed that *pdgfba* and *pdgfrb* were significantly upregulated over baseline in $CoCl_2$ treated embryos, whereas related family members were not affected (Tx 12-36hpf) (*p<0.05, two-tailed t-test, n≥3).

(E) In *vhl* MO injected embryos RT-qPCR analysis likewise indicated that only *pdgfba* and *pdgfrb* were significantly upregulated over matched controls (Tx 12-36hpf) (*p<0.05, **p<0.01, two-tailed t-test, n≥4).

(F) WISH analysis of wild-type embryos at 36hpf indicated *pdgfrb* expression throughout the trunk of the embryo, including enrichment in the region of the VDA; *pdgfrb* expression was notably increased by $CoCl_2$ exposure.

(G) Qualitative phenotypic distribution of embryos from panel 1F scored with low, medium or high *pdgfrb* expression in the AGM (n≥20 condition × 2 replicate clutches).

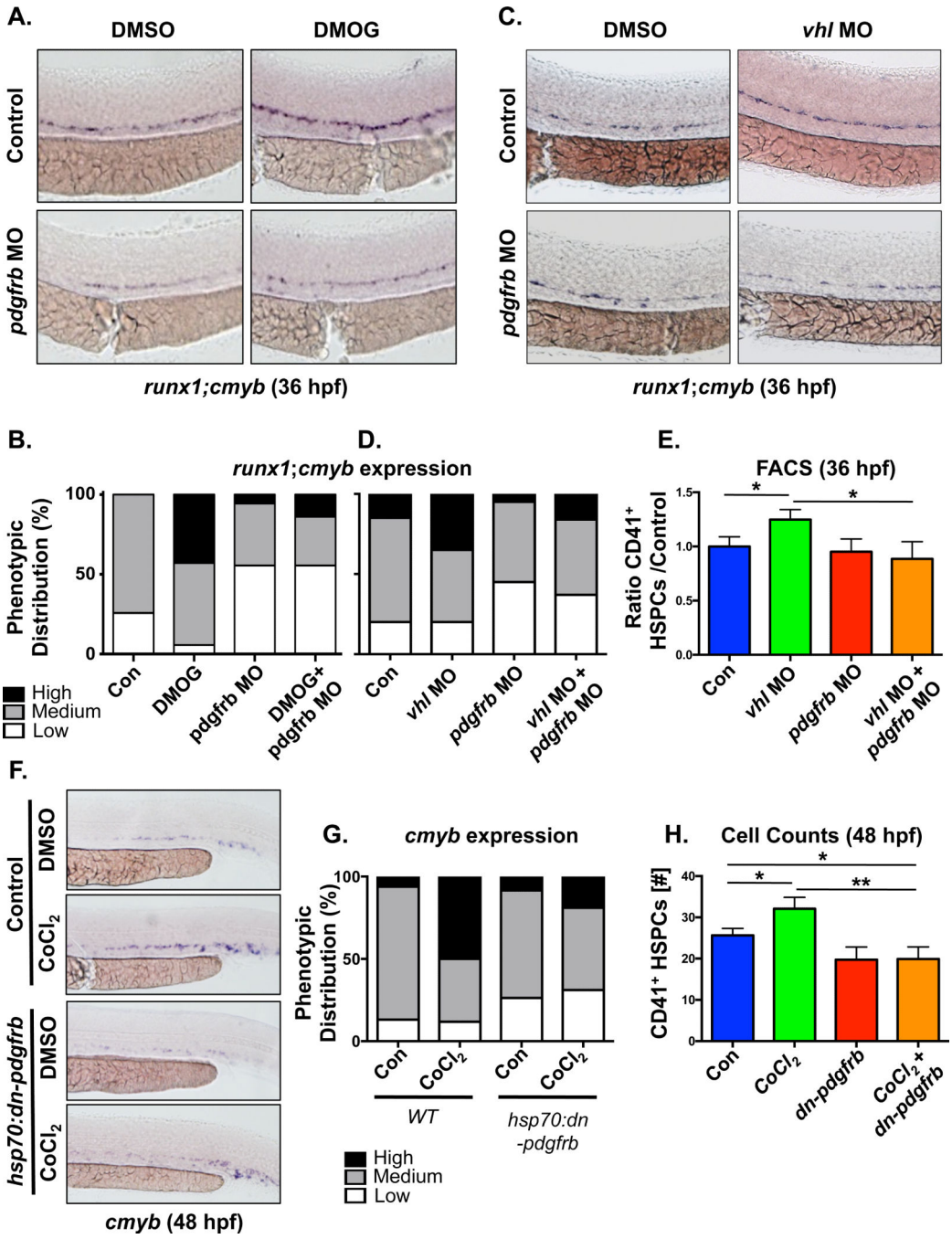

Fig 2. PDGFRβ signaling acts downstream of Hif1α to control AGM HSPC production

(A) Morpholino knockdown of *pdgfrb* attenuated the increase in *runx1;cmyb* expression in embryos treated with the Hif1α agonist DMOG (75μM).

(B) Qualitative phenotypic distribution of embryos from panel 2A (n≥20/condition × 3 replicate clutches).

(C) Knockdown of *vhl* increased *runx1;cmyb* WISH expression in the AGM at 36hpf, while co-injection with the *pdgfrb* MO blocked this effect.

(D) Qualitative phenotypic distribution of embryos from panel 2D (n≥20/condition × 2 replicate clutches).
(E) FACS analysis for $CD41^{+}(Gata1^{-})$ HSPCs following injection of MOs to *vhl* and *pdgfrb* alone and combined confirmed a reduced impact for Hif1α stabilization with loss of PDGF signaling (*p<0.05, one-tailed t-test, n≥7).
(F) Ectopic activation of a dominant negative *pdgfrb* transgene, Tg(*hsp70:dn-pdgfrb),* at 27hpf (37°C for 1 hour) diminished the impact of $CoCl_2$ stimulation on *cmyb* expression at 48hpf.
(G) Qualitative phenotypic distribution of embryos from panel S2G (n≥20/condition × 4 replicate clutches).
(H) $CD41^{+}$ HSPC cell counts at 48hpf confirmed the effect of loss of PDGFRB signaling, mediated by induction of a dominant negative receptor, on Hif1α activation by $CoCl_2$ (*p<0.05, **p<0.01, one-tailed t-test, n≥8).

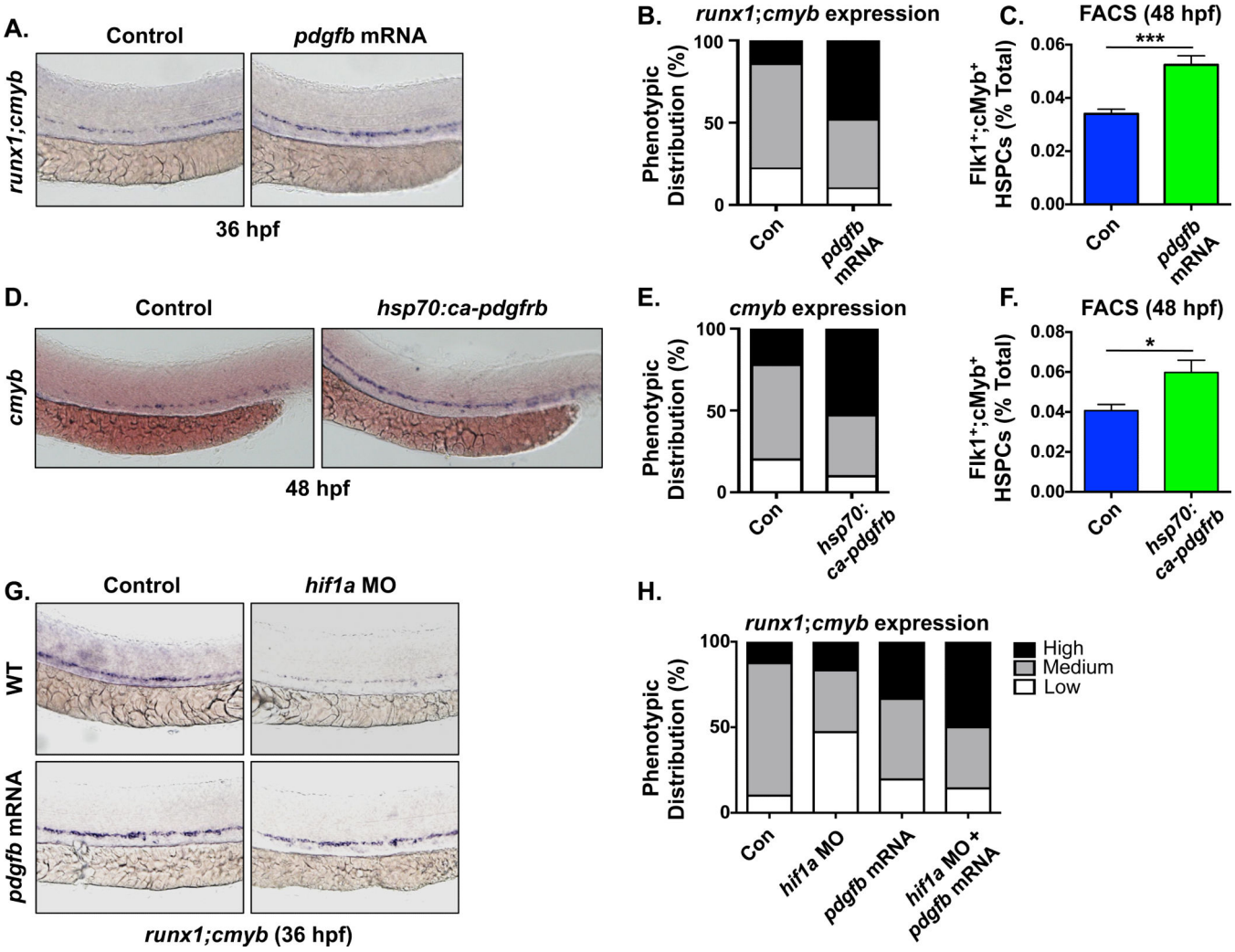

Fig 3. PDGF-B stimulation increases developmental HSPC numbers

(A) Overexpression of *pdgfb* by mRNA injection enhanced *runx1;cmyb* expression in the VDA by WISH at 36hpf.

(B) Qualitative phenotypic distribution of embryos from panel 3A (n≥20/condition × 5 replicate clutches).

(C) FACS analysis confirmed that *pdgfb* overexpression increased Flk1:dsRed^{+}cMyb:GFP^{+} HSPCs at 48hpf (1.54 fold increase, ***p≤0.0001, two-tailed t test, n≥5 replicates/condition).

(D) WISH analysis showed enhanced *cmyb* expression at 48hpf following ectopic activation of PDGFRβ signaling using *hsp70:ca-pdgfrb* induced at 27hpf (37°C for 1 hour)

(E) Qualitative phenotypic distribution of embryos from panel 3D (n≥20/condition × 5 replicate clutches).

(F) FACS analysis confirmed a significant increase in Flk1^{+}cMyb^{+} HSPCs (48hpf) following PDGFRβ activation mediated by induced expression of *ca-pdgfrb* (1.47 fold increase, *p<0.05, two-tailed t test, n≥5 replicates/condition).

(G) Overexpression of *pdgfb* ligand rescued the reduction in *runx1;cmyb* expression found in *hif1a* morphants at 36hpf.

(H) Qualitative phenotypic distribution of embryos from panel 3G (n≥20/condition × 3 replicate clutches).

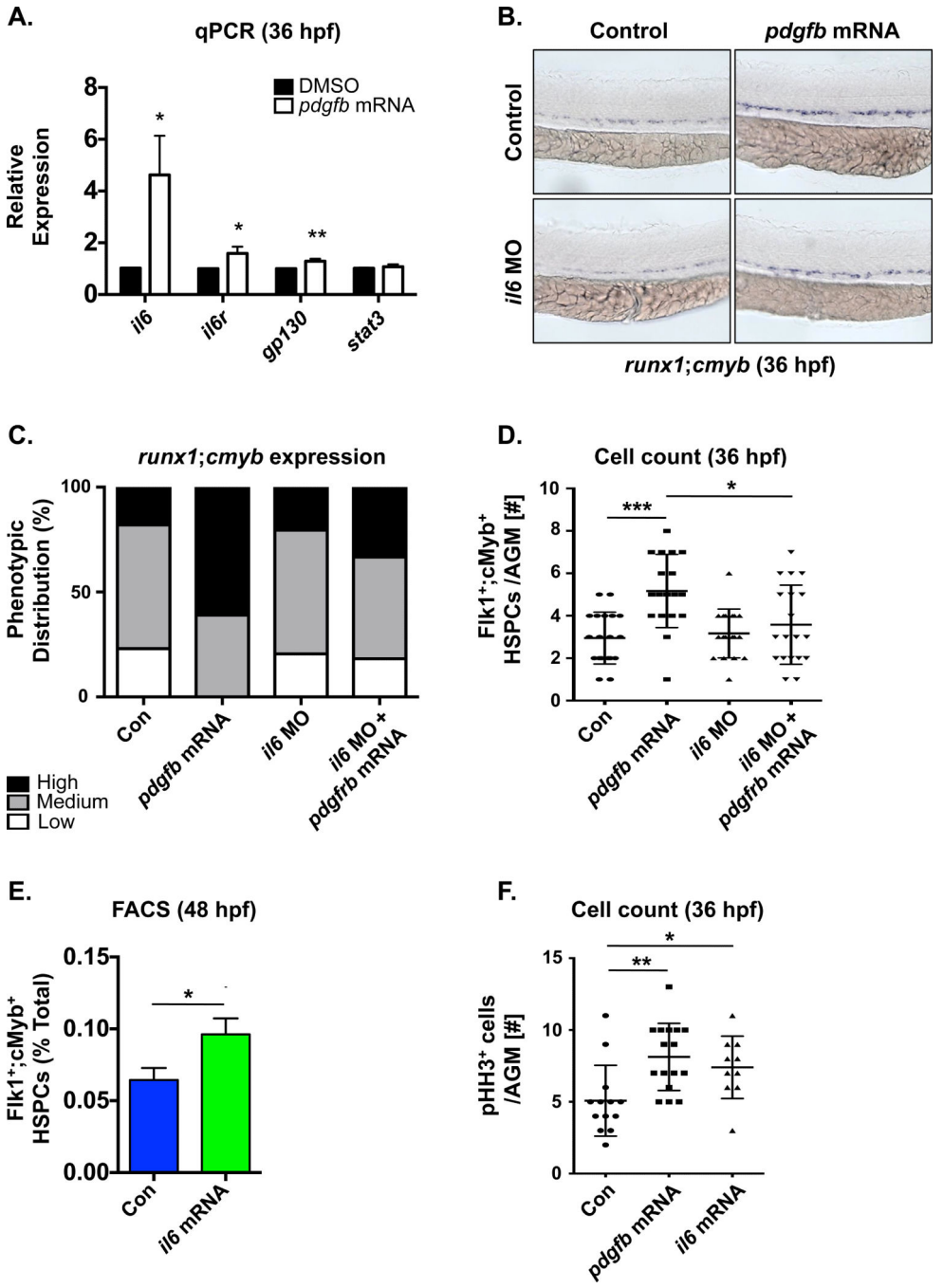

Fig 4. IL-6 acts downstream of PDGFRβ signaling to stimulate AGM HSPC production

(A) qPCR analysis showed *il6* and its' receptor and co-receptor, *il6R* and *gp130*, are upregulated in *pdgfb* mRNA-injected embryos (*p<0.05, **p<0.01, two-tailed t-test, n≥3).

(B) Morpholino knockdown of *il6* blocked the ability of *pdgfb* mRNA to increase *runx1;cmyb* expression in the AGM.

(C) Qualitative phenotypic distribution of embryos from panel 4B (n≥20/condition × 3 replicate clutches).

(D) Absolute counts of Flk1:dsRed^{+}cMyb:GFP^{+} HSPCs from embryos overexpressing *pdgfb* were significantly reduced with MO-mediated loss of *il6* (***p<0.0005, *p<0.05, two-tailed t-test, n≥15/condition).

(E) FACS analysis for Flk1^{+}cMyb^{+} HSPCs at 48hpf showed that overexpression of *il6* significantly increases HSPCs (1.49-fold vs. control, *p<0.02, two-tailed t-test, n≥5 replicates/condition).

(F) Absolute cell counts of phospho histone H3 expressing (pHH3^{+}) cells in the VDA were increased in both *pdgfb* and *il6* mRNA-injected embryos compared to matched sibling controls (*p<0.05, **p<0.01, two-tailed t-test, n ≥10/condition).

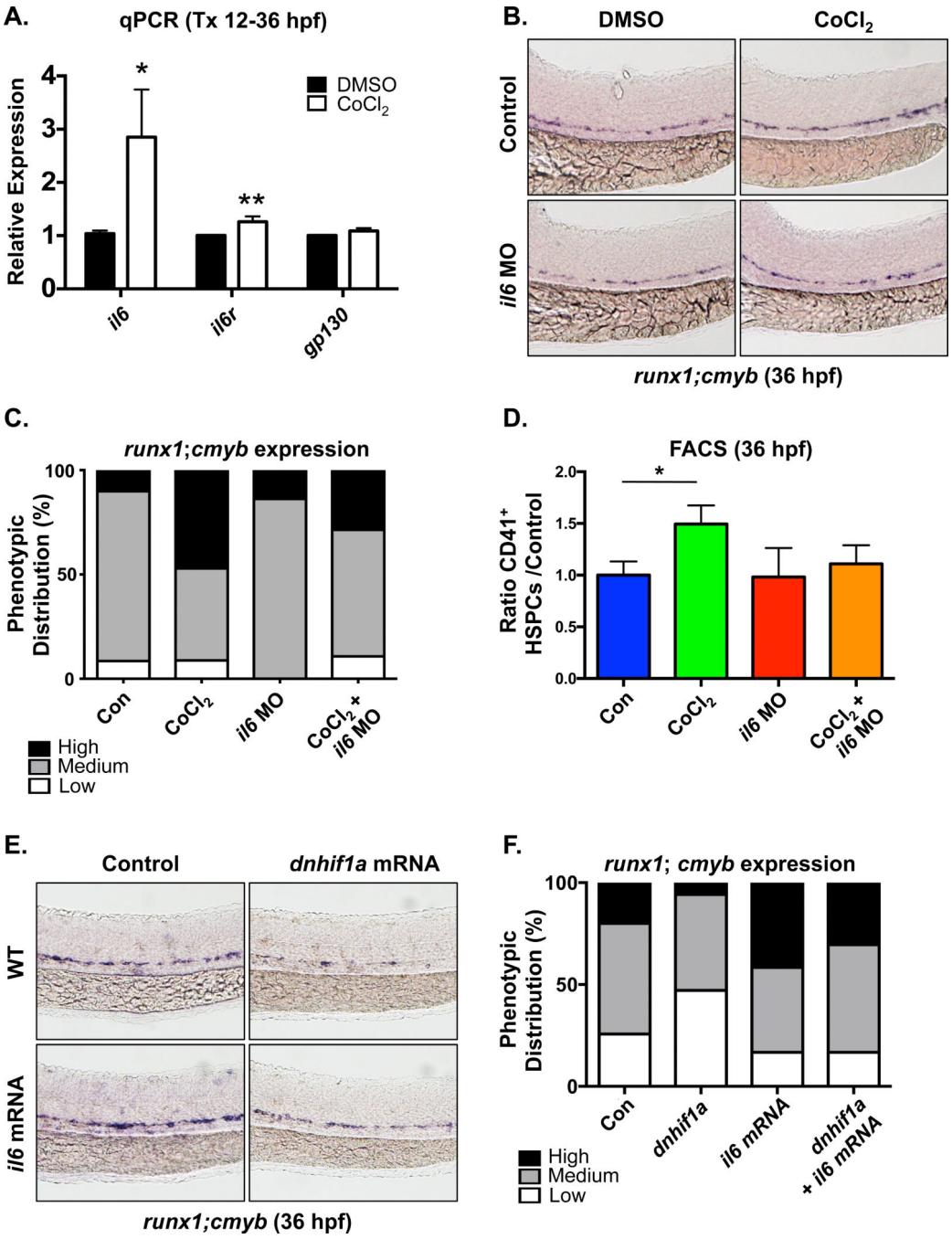

Fig 5. IL-6 signaling functions downstream of Hif1α in AGM HSPC regulation

(A) RT-qPCR analysis demonstrated that expression of *il6* and its receptor *il6R* are increased by $CoCl_2$ exposure (*p<0.05, **p<0.01, one-tailed t-test, n≥3).

(B) Morpholino knockdown of *il6* diminished the ability of $CoCl_2$ to stimulate *runx1;cmyb* expression in the VDA over that seen in controls as determined by WISH analysis at 36hpf.

(C) Qualitative phenotypic distribution of embryos from panel 5B (n≥20/condition × 3 replicate clutches).

(D) FACS analysis confirmed that $CoCl_2$ was unable to enhance the number of $CD41^+$ HSPCs in the presence of *il6* knockdown (*p<0.05, one-tailed t-test, n≥4).
(E) Injection of *dnhif1a* mRNA decreased *runx1;cmyb* expression in the VDA at 36hpf, which could be partially ameliorated by overexpression of *il6.*
(F) Qualitative phenotypic distribution of embryos from panel 5E (n≥20/condition × 3 replicate clutches.