PDCD2 Controls Hematopoietic Stem Cell Differentiation During Development

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Programmed cell death 2 (*Pdcd2*) is a highly conserved protein of undefined function, and is widely expressed in embryonic and adult tissues. The observations that knockout of *Pdcd2* in the mouse is embryonic lethal at preimplantation stages, and that in *Drosophila*, *Zfrp8*, the ortholog of *Pdcd2*, is required for normal lymph gland development suggest that *Pdcd2* is important for regulating hematopoietic development. Through genetic and functional studies, we investigated *pdcd2* function during the zebrafish ontogeny. Knockdown of *pdcd2* expression in zebrafish embryos resulted in defects in embryonic hematopoietic development. Loss of *pdcd2* function caused increased expression of progenitor markers, and accumulation of erythroid progenitors during primitive hematopoiesis. Additionally, hematopoietic stem cells (HSCs) failed to appear in the aorta-gonad mesonephros, and were not able to terminally differentiate or reconstitute hematopoiesis. *Pdcd2* effects on HSC emergence were cell autonomous and *P53*-independent, and loss of *pdcd2* function was associated with mitotic defects and apoptosis. Restoration of *runx1* function(s) and modulation of apoptosis through the inhibition of Jak/Stat signaling rescued the hematopoietic and erythroid defects resulting from *pdcd2* knockdown. Our studies suggest that *pdcd2* plays a critical role in regulating the transcriptional hierarchy controlling hematopoietic lineage determination. Furthermore, the effects of *pdcd2* in regulating mitotic cell death may contribute to its role(s) in directing hematopoietic differentiation during development.

Introduction

IN ALL VERTEBRATES FROM zebrafish to human, hematopoiesis is a dynamic process that occurs in successive stages and at distinct anatomic sites [1]. The successive hematopoietic waves are directed by morphogenetic movements, and by expression of transcription factors that regulate lineage commitment and cellular reprogramming [2]. In mammals, primitive hematopoiesis is characterized by generation of primitive macrophages and erythroid progenitors in the extraembryonic yolk sac. In zebrafish, this first hematopoietic wave occurs in 2 distinct regions in the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM) [2]. The bilateral stripes of cells in the PLM later converge at midline to form the intermediate cell mass (ICM), the zebrafish equivalent to mammalian yolk sac blood islands at the 20-somite stage or ~19-hour postfertilization (hpf) [2]. The specification of PLM-derived cells into hematopoietic lineages marked by *scl*, *gata2*, and *lmo2*, and endothelial lineages marked by *flk1* and *fli1* occurs before the morphogenetic movement of the PLM to form the ICM [3,4]. PLM-derived cells later differen-

tiate into proerythroblasts and endothelial cells in the trunk vasculature. Within the ICM, proerythroblasts that are *gata1*⁺ enter the circulation between 24–26 hpf, and subsequently mature into primitive erythrocytes.

A transient wave between primitive and definitive hematopoiesis is reported to occur between 1 and 2 days post-fertilization (dpf) in the zebrafish posterior blood island, and is characterized by the appearance of bipotential erythromyeloid progenitors (EMP) [5]. In zebrafish as well as in the mouse, the close association between hematopoietic cells and the developing endothelium suggests that both lineages arise from either a mesodermal progenitor(s) with endothelial and hematopoietic potential called the hemangioblast [2], or from the hemogenic endothelium. Using single-cell labeling and live-cell imaging, hemangioblasts have been reported in zebrafish [6], and differentiation of hematopoietic cells from the hemogenic endothelium was demonstrated [7–9]. The 2 views are not mutually exclusive and it has been proposed that *flk1*⁺ hemangioblasts first generate the hemogenic endothelium in a step that depends on *scl* expression, and then produce definitive hematopoietic cells through *runx1* expression [10].

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Multipotent definitive hematopoietic stem cells (HSCs) that express *runx1* and *c-myb* [2], and are capable of self-renewal and generation of all definitive blood lineages first emerge from a region between the dorsal aorta and the posterior cardinal vein equivalent to the aorta-gonad mesonephros (AGM) region in mammals [11,12]. From 48 hpf, *c-myb*⁺ cells are found in the posterior region in the tail called the caudal hematopoietic tissue (CHT) [13,14], a site equivalent to mammalian fetal liver. These *c-myb*⁺ cells later appear in the thymus from 3 dpf and in the kidney marrow from 4 dpf [13,14], the sites for adult hematopoiesis in larval and adult zebrafish.

Runx1 is a key regulator of definitive hematopoiesis in all vertebrates, including zebrafish [11,12]. *Runx1* expression modulates both the efficiency of HSC emergence from the hemogenic endothelium as well as the survival of these HSCs [8,15]. The formation of HSCs expressing *runx1* and *cmyb* is known to require blood flow [16]. Further, hematopoietic differentiation is regulated by equilibrium between downstream transcription factors, such as *gata1* and *pu.1* that regulate myeloid versus erythroid progenitor cell fate [17], where *gata1* promotes erythroid differentiation [18], while *pu.1* marks early myelopoiesis and promotes myeloid differentiation [19].

Clearly, many important regulators of hematopoiesis and HSC function remain to be described. Programmed cell death 2 (*Pdcd2*) was initially identified among genes upregulated in rat thymocytes triggered to undergo apoptosis by irradiation or dexamethasone treatment [20]. Studies in *Drosophila* suggested that *pdcd2* plays an important role in hematopoiesis [21]. Mutations in the *Drosophila* ortholog of *pdcd2*, *zfrp8*, resulted in hyperplasia of the fly lymph gland, and *zfrp8* was subsequently shown to be essential for maintaining the stem cell population, but dispensable for their daughter progenitor cells [21,22]. Interestingly, transcriptional profiling of various stem cells identified *Pdcd2* among genes enriched in mouse embryonic, neural, and HSCs [23]. *Pdcd2* is highly expressed in mouse blastocysts [24], and is required for ESC maintenance [25]. *PDCD2* is also enriched in human ESCs compared to their differentiated lineages [26]. Human *PDCD2* is located on chromosome 6q27 in a region involved in both translocations and deletions in leukemias and lymphomas [27]. This involvement suggests that *PDCD2* is a potential tumor suppressor gene [28] that might be required for myeloid and/or lymphoid cell fate.

Little is known of the function of *PDCD2*, although it has been shown to physically interact with the host cell factor-1 (*hcf1*) that controls cell cycle progression, and to bind to N-CoR through its MYND domain implying a possible role in transcriptional regulation [29]. We hypothesized that *pdcd2* is required for normal hematopoiesis in zebrafish. We demonstrate that loss of *pdcd2* perturbs embryonic hematopoietic development, and this phenotype was associated with cell cycle defects and apoptosis, demonstrating a critical role for *pdcd2* in regulating hematopoietic development.

Materials and Methods

Fish maintenance

The transgenic zebrafish *Fli1:EGFP* and *gata1:dsRed*, and the *P53* mutant zebrafish lines were purchased from the zebrafish international resource center (ZIRC) (University of

Oregon). Transgenic *CD41:EGFP* zebrafish were previously described [30]. Zebrafish were maintained at 28.5°C in a recirculating aquaculture system at the zebrafish facility of Cancer Institute of New Jersey according to guidelines established at UMDNJ.

mRNA synthesis, morpholinos, and microinjection

Capped *pdcd2* mRNA was synthesized using the mMessage mMachine kit (Ambion). RNA synthesis for in situ probes was performed using either digoxigenin- or Fluorescein-UTP (Roche). Morpholino oligonucleotides (MO; Gene Tools LLC) were ATG-MO (5'-TGTATTTGCAGCCATCTGTCAAACCT-3'), which targets the 5'UTR of *pdcd2*, and 5ssMO (5'-TAA CATCTGTTTTGCATCACCTGGA-3'), which targets splicing of intron 5. Control MO had 5 mismatched nucleotides to the ATG-MO displayed in lower cases (5'-TGTAaTtcCAGCg-ATCTGTgAAtCT-3'). Each MO was injected into 1–2 cell embryos after titration at 5.5, 2, and 5.5 ng/embryo, respectively. A *P53* MO (5'-GCGCCATTGCTTTGCAAGAATTG-3') was used at 4.5 ng/embryo.

Whole-mount in situ hybridization, histology, and immunohistochemistry

RNA whole-mount in situ hybridization (WISH) for zebrafish *pdcd2*, *scl*, *c-myb*, *pu.1*, *gata1*, *runx1*, *lmo2*, *rag1*, *hbae1*, *mpo*, *l-plastin*, and *lck* were performed as described [31]. Immunohistochemistry (IHC) for human, mouse, and zebrafish *PDCD2* was performed as described [32] using a polyclonal anti-*PDCD2* antibody (1:500; Proteintech). Mouse embryo collections, mouse *Pdcd2* IHC, and 2,7-diaminofluorene (DAF) labeling for zebrafish were performed as described (Supplementary Data are available online at www.liebertpub.com/scd). Phospho-histone H3 (p-H3) was detected using a polyclonal anti-p-H3 antibody (1:750; Millipore), the Alexa-555 secondary antibody (Invitrogen), and DAPI counterstaining. Periodic Acid Schiff (PAS) staining (Sigma) and Sudan Black labeling were performed as described [33]. Apoptosis assays and BrdU labeling were performed as described [34].

Flow cytometry, fluorescent activated cell sorting, and cytology

Embryos were prepared for cytometry as described ([35], and Supplementary Data). Cell sorting of *gata1:DsRed* embryos was performed using a BD cell sorter, and transplantation was performed as described [16]. For cytology, *DsRed*⁺ cells were spun for 3 min at 300 rpm using a cytospin3 centrifuge (Shandon), and slides were stained with May-Grunwald Giemsa.

Generation of transgenic *pdcd2* reporter zebrafish

Transgenesis was achieved by injection of *pdcd2* (–1.6 kb): *AcGFP* in one-cell stage embryos as described ([32], and Supplementary Data). Multiple founder lines were established based on enhanced green fluorescent protein (*EGFP*) expression. RNA preparations for RT-PCR were done using the RNAqueous kit (Ambion). Oligo-dT primed cDNA from embryonic stages and tissues were used for PCR using *pdcd2*-, *runx1*-, and β -actin-specific primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/scd).

Results

Identification of the zebrafish *pdc2* gene and putative regulatory elements

We found the zebrafish *pdc2* gene [zgc:63685, designated as ENSDARG0000007892 in Ensembl (zv8)] to be composed of 6 exons spanning 3,766 bp encoding a 358 aa protein (~47 kDa), which shares significant homology, a central MYND, and a C-terminal PDCD2 domain with all *pdc2* orthologs (Supplementary Fig. S1A, D). Phylogenetic analysis using the PDCD2 domain suggests that *pdc2* is conserved among all eukaryotes (Supplementary Fig. S1A–D). Zebrafish *pdc2* is located on chromosome 13, and despite a smaller zebrafish chromosome size, both *pdc2* and the syntenic neighboring gene *tbp* are conserved (Supplementary Fig. S1C). To identify the zebrafish *pdc2* promoter and subsequently characterize the *in vivo* expression of *pdc2*, we performed *in silico* promoter and transcription factor-binding site (TFBS) analyses. A minimal 1.6-Kb core promoter region was identified, and included a proximal 800-bp region con-

taining over 500 TFBS, including factors regulating hematopoiesis, such as *runx1*, *gata* factors, *c-myb*, *ap1*, *c/ebp*, *scl/tal1*, *p53*, *lmo2*, *ets* factors, and *stat* (data not shown). We utilized this zebrafish *pdc2* promoter to drive *EGFP* expression in a transgenic reporter line, and the expression of *EGFP* in these transgenic embryos was detected ubiquitously throughout embryonic development (Supplementary Fig. S2A, B). Importantly, *EGFP* was detected in circulating blood cells in the stable transgenic *pdc2* (–1.6 kb):*EGFP* embryos at ~26 hpf (Supplementary Movie S1).

Expression of *pdc2* in human, mouse, and zebrafish during development

As noted above, it has been suggested that PDCD2 plays a role in development and hematopoiesis. We therefore investigated *pdc2* expression in human, mouse, and zebrafish tissues using IHC and RNA WISH. *Pdc2* expression is ubiquitous and expressed in hematopoietic tissues in normal human bone marrow (BM) (Fig. 1A, B), mouse fetal liver (Fig. 1C–E), and zebrafish embryonic stages (Fig. 1F–H).

FIG. 1. PDCD2 expression in human, mouse, and zebrafish tissues. IHC of PDCD2 expression in human BM (A, B), mouse fetal liver (C–E), and zebrafish embryos (F–H). (B) is a higher magnification of outlined area in (A). Notice the dense nuclear and cytoplasmic staining in progenitor-like cells (arrow) compared with a lighter staining in surrounding differentiated cells, and in a megakaryocyte (arrowhead). (C) IHC of *Pdc2* expression in a sagittal section of an E13.5 mouse embryo showing the fetal liver (outlined area) in (C), and higher magnifications in (D) and (E). Strong *Pdc2* expression is seen in hematopoietic and dividing cells (arrows in E), but not in cells lining the liver sinusoids (arrowhead in E). *Pdc2* protein expression by IHC of zebrafish embryos with strong ubiquitous expression in early embryonic stages at 18 hpf (F), 24 hpf (G), and 36 hpf (H). Insets are magnifications of the outlined areas in G and H. Arrows point to hematopoietic cells in the ICM and AGM regions. Images are lateral view, anterior to the left, and dorsal upward. Scale bars are 100 μ m in (A, F, and G), 20 μ m in (B, E, and H), and 500 μ m in (C, D, and F). Regions outlined include the AGM, and the tail region that would latter contain the CHT/PBL. nc, notochord; yt, yolk tube; DA/AV, dorsal aorta and axial vein region; AGM, aorta-gonad mesonephros; BM, bone marrow; CHT, caudal hematopoietic tissue; hpf, hour postfertilization; ICM, intermediate cell mass; IHC, immunohistochemistry; *Pdc2*, programmed cell death 2. Color images available online at www.liebertpub.com/scd

Using WISH of developing zebrafish embryos, *pdc2* mRNA was found to be maternally expressed at the 4-cell stage (Supplementary Fig. S3A), and widely distributed during gastrulation and somitogenesis (Supplementary Fig. S3B–F). In zebrafish embryos, *pdc2* mRNA was detected by RT-PCR during the first 5 days of embryonic development (Supplementary Fig. S3G). Strong *pdc2* expression was detected in the ICM (Fig. 1G), and at the AGM region, the site of HSC formation at later stages (Fig. 1H). In adult fish, *pdc2* expression was also detectable in all tissues examined except muscle, with a substantial enrichment in kidney marrow (Supplementary Fig. S3H). Collectively, *pdc2* expression analyses in mammals and zebrafish suggest that while *pdc2* is expressed ubiquitously, it is highly enriched in both neural and hematopoietic tissues.

Pdc2 knockdown during zebrafish embryonic development

We assessed zebrafish development after *pdc2* site-directed knockdown using *pdc2*-specific MO. We used mismatched MOs as controls, and 2 *pdc2*-specific MOs: ATGMO, which targets the translational start codon, and 5ssMO, which represses the splicing of intron 5 of *pdc2* (Supplementary Fig. S4A).

Western blotting using the anti-*pdc2* antibody and RT-PCR analyses revealed reductions in the *pdc2* protein, or aberrant *pdc2* splicing in ATGMO- or 5ssMO-injected embryos, respectively (Supplementary Fig. S4B, C). Given the widespread *pdc2* expression, we anticipated that *pdc2* knockdown would produce severe developmental defects. However, *pdc2* ATGMO-injected embryos had no apparent defects up to late somitogenesis. Starting at ~18 hpf and up to 48 hpf, we noted that ATGMO-injected embryos displayed smaller eyes, blocky rather than chevron-shaped somites, dilated brain ventricles, oblong-shaped yolk with shortened yolk tube, and curly and thickened tails compared to control embryos (Fig. 2A–D). None of the mismatch MO-injected embryos displayed this phenotype. Injections of the *pdc2* splice 5ssMO reproduced these defects at significantly lower doses (2 ng for 5ssMO vs. 5.5 ng for ATGMO). These phenotypes were seen in ~90% of ATGMO- or 5ssMO-injected embryos. Given that 5ssMO was most efficient in repressing *pdc2* expression (Supplementary Fig. S4C), we used the 5ssMO in most of our studies.

Loss of *pdc2* impairs primitive erythropoiesis

In zebrafish embryos, primitive erythroid cells undergo synchronized intravasation and release from vessel walls into the circulation at 23–26 hpf [36]. *Pdc2* ATGMO- and 5ssMO-injected embryos (*pdc2* morphants) exhibited a dose-dependent failure in this process and only small numbers of erythrocytes were observed in circulation even at 30 hpf ($n \geq 500$ embryos). We attempted to establish a dose-response curve to *pdc2* knockdown by examining the numbers of erythrocytes in circulation through different components of the embryonic vasculature at 48 hpf (Fig. 2E). We observed a dose-dependent effect of *pdc2* knockdown in *pdc2* morphant embryos with high doses, resulting in complete absence of circulating erythrocytes in both axial and intersegmental vessels. Importantly, these effects were

only seen in *pdc2* ATGMO- and 5ssMO-injected, but not in mismatch MO-injected embryos, and were rescued by the injection *pdc2* mRNA (Fig. 2E), suggesting that the hematopoietic phenotype is specific to *pdc2* knockdown. Circulation through intersegmental vessels appeared to be most sensitive to *pdc2* knockdown with subtle patterning defects in the number and position of intersegmental vessels. The release of primitive erythrocytes into the circulation must be tightly coordinated with vascular organization and contractions of the primitive heart tube [37,38]. Thus, the hematopoietic circulatory defects in *pdc2* morphants might be attributable to defects in cardiac or vascular development, or due to the presence of defective primitive hematopoietic cells.

Circulating cells during early embryogenesis are comprised mostly of primitive erythrocytes and a few primitive macrophages [2]. Thus, we investigated the effects of *pdc2* knockdown in *gata1:DsRed* transgenic fish [35] with DsRed-labeled erythrocytes. The initial defects were morphologically visible at onset of circulation when DsRed⁺ cells from *gata1:DsRed pdc2* morphants showed delayed egress from the ICM (Fig. 2F–H). At a time when control embryos exhibited release of circulatory erythrocytes (from 26 hpf onward), very few erythrocytes were observed to be actively circulating in *pdc2* morphant embryos (Supplementary Movies S2 and S3). Exit of most erythrocytes from the ICM had occurred in >90% of the embryos by 36 hpf, and *gata1:DsRed*⁺ cells were observed to accumulate in the yolk vascular sinuses thereafter ($n=34$; data not shown). By 48 hpf, control embryos exhibited robust numbers of circulating primitive erythrocytes at the duct of Cuvier and trunk vessels, while *gata1:DsRed*⁺ cells from *pdc2* morphants remained pooled at the yolk vascular sinuses, and only occasional DsRed⁺ cells were observed in the trunk vessels and within the interstitial space coincident with the former ICM (Fig. 2J, K, Supplementary Movies S4 and S5). Thus, *pdc2* knockdown results in aberrant erythroid cell movement with limited numbers of cells intravasating into the circulation without the synchrony apparent in control embryos (Compare Supplementary Movie S5 to control in Supplementary Movie S4). Moreover, once within the vasculature, *pdc2* morphant erythrocytes fail to circulate freely, and the small number of circulating erythrocytes present at 48 hpf occasionally traversed the trunk vasculature.

We sought to determine whether the effects upon erythroid cell movements were a secondary consequence of an underlying cardiac or vascular defect. Both light microscopic analyses and microangiography indicate that heart function is not grossly perturbed (Fig. 3A, B). *Pdc2* morphants displayed regular cardiac development and contractions of the primitive heart tubes. Heart rates were 98 ± 4 beats/min in *pdc2* morphants compared to 100 ± 3 beats/min in control MO-injected embryos, and 99 ± 4 beats/min in WT embryos at 32 hpf, which are comparable to previously reported values [37]. Additionally, microangiography with fluoresceinated carboxylated latex beads [39] indicated that *pdc2* morphants had patent vasculature that exhibits plasma flow (compare Fig. 3A–A' and 3B–B').

To further assess any potential fine perturbations in vascular development, we examined the labeled vasculature in the *Fli1:EGFP* transgenic embryos with reduced *pdc2* function. Observations of embryos spanning the developmental

FIG. 2. Developmental defects in primitive hematopoiesis in *pcd2* morphants. (A–D) Bright field images of control (A, C) and *pcd2* morpholino injected embryos (B, D) at 18 hpf (A, B) and 48 hpf (C, D). *Pcd2* morphants displayed significantly smaller eyes (arrow), a slightly shorter axis, dilated brain ventricles (*) with brain necrosis, and curly and thickened tails at both 18 and 48 hpf (**). (D). The yolk appeared oblong in shape due to accumulation of hematopoietic cells at the lower anterior part of the yolk sac. These phenotypes were seen in nearly 90% of >5,000 ATGMO- or 5ssMO-injected *pcd2* morphant embryos. (E) Scoring system based on the numbers of blood cells released to the circulation after injection of control-mismatch MO, or *pcd2* 5ssMO alone or combined with *pcd2* mRNA to rescue the phenotype. Embryos were classified into 3 classes based on their flowing blood cell numbers: (i) indistinguishable from the control (normal) as indicated by the gray bar, (ii) cell number reduction (<30 cells transiting the axial or intersegmental vessels) (reduction) compared to control as indicated by the red bar, and (iii) severely deficient or almost no flowing blood cells (<10 cells transiting the axial or intersegmental vessels) (deficiency) compared to control as indicated by the blue bar (Bars—s.e.m., *three 5ssMO doses are significantly different than control, mRNA rescue of 5ssMO effects were significantly different than 5ssMO alone, $P < 0.01$). (F–K) Representative fluorescent DsRed images of control MO- (cont.), 5ssMO-, or ATGMO-injected *gata1:DsRed* embryos at 24 or 48 hpf showing failure of hematopoietic cell release. Primitive erythrocytes have cleared the ICM and entered the major vessels (outlined rectangle) and yolk circulation (outlined circle) in 24 hpf control embryos (F), while in *pcd2* morphant embryos (G, H), cells appear to remain in the ICM region. *Gata1:DsRed*⁺ cells are distributed throughout the circulatory system at 48 hpf in control embryos (I) partitioned between the yolk's duct of Cuvier (I') and the vasculature (e.g., trunk vessels in I''). DsRed⁺ cells in *pcd2* morphant embryos (J, K) accumulate at the duct of Cuvier (J', K') and fail to continuously release into the interstitial and major trunk vessels (J'', K''). Data represent 6 independent experiments utilizing ~100 embryos/variable, and phenotypes were seen in nearly 90% of control MO-, ATGMO-, or 5ssMO-injected *pcd2* morphant embryos. MO, morpholino oligonucleotides; s.e.m., standard error of the mean. Color images available online at www.liebertpub.com/scd

period during which the embryonic vasculature is established indicated that the major axial vessels in the trunk appear to develop normally (Fig. 3C, D). The number of EGFP⁺ cells detected in morphant embryos was similar to that observed in controls, indicating that the expression of EGFP from the *flil* promoter was unaffected by *pcd2* knockdown (compare Fig. 3E–H). Similar to the effect of *pcd2* knockdown on erythrocytes, *Flil:EGFP* morphants displayed an accumulation of circulatory cells at the yolk's duct of Cuvier (data not shown). Endothelial expression of EGFP cells in *Flil:EGFP* morphants indicates that the major vessels of the trunk, including the dorsal aorta and caudal

vein, were established at about the same developmental timing as seen in control embryos (Fig. 3E–H and data not shown). Arterial specification occurred, and mature endothelial cells expressing ephrinb2a (*ephrb2a*) were not affected in *pcd2* morphants (Fig. 3I, J). Further, the expression of the VEGF receptor *flk1* in endothelial sites was comparable between *pcd2* morphants and controls (Fig. 3K–N). Intersegmental vessels and the dorsal axial vein appeared to sprout and form fairly normally. *Pcd2* morphants displayed array of intersegmental vessels that are lacking the chevron-shaped path exhibited by control intersegmental vessels. However, this is likely a reflection of the blocky nature of somite

FIG. 3. Analysis of vasculogenesis in *pdc2* morphant embryos. Microangiography of control (A, A') and 5ssMO-injected wild-type embryos (B, B') injected with fluorescein-labeled beads at 48 hpf in the anterior yolk sinus. Images collected within 30 min of injection indicate that the major vessels as well as the smaller ancillary vessels of the trunk are patent and permit blood flow. (C, D) H&E histological sections of the mid-trunk region of control MO-injected (C) and 5ssMO-injected (D) zebrafish embryos. The sections demonstrate the presence of comparable endothelial cell thickness and lumen size of major trunk vessels (black arrows). NT, neural tube; DA, dorsal aorta; AV, axial vein. (E–H) Analysis of dose-dependent effects of *pdc2* knockdown on vascular development in *flil:EGFP* embryos shown at 48 hpf. Injection of control or 5ssMO *pdc2* MO had no discernable effect on the presence of major vessels (DA and AV) in the trunk or head (E–H, E'–H' and data not shown). Intersegmental vessels were present at intersomitic boundaries and appear to be slightly thicker in *pdc2* morphants than in controls (arrows). This may be a consequence of the deformed nature of the somites discussed earlier (Fig. 2B). Gaps in the dorsal axial vein (DLAV) represent regions adjacent to the posterior tail somites that were irregular at higher morpholino inputs (arrowheads). (I, J) WISH of mature endothelial cell marker; ephrin (*ephrb2a*) in 5ssMO-injected embryos (J) compared to controls (I) at 26 hpf (controls, $n=26/26$ (100%) showed the displayed phenotype; 5ssMO, $n=24/25$, 96% showed the displayed phenotype). Arterial specification occurred, and levels of *ephrb2a*⁺ cells (arrowheads in inset) were comparable between control and 5ssMO-treated embryos. (K–N) Expression of the generalized endothelial cell marker, *flk1*, was comparable at 26 hpf between control (K, M) and *pdc2* knockdown embryos (L, N) (control; $n=36$ (97%) showed the displayed phenotype, 5ssMO; $n=47$, 89% showed the displayed phenotype). (O, P) Formation of the caudal venous plexus (dashed boxes O, control and P, 5ssMO) in *pdc2* morphants at 72 hpf was defective as visualized in *Fli1:EGFP*. ISV, intersegmental vessels; AC, axial circulation; EGFP, enhanced green fluorescent protein; WISH, whole-mount in situ hybridization. Color images available online at www.liebertpub.com/scd

boundaries in morphants that delineates the path that these vessels follow, and it is unlikely a consequence of any intrinsic angiogenic defect (see Fig. 3A–H). One notable difference in morphant embryos was the formation of the caudal venous plexus. Formation of this anastomosing network of vasculature is reported to begin at ~32 hpf, and is believed to serve as a niche for both hematopoietic precursor and stem cells [40]. *Pdc2* morphants displayed a defect in the formation of this vascular network that became more apparent at later stages and may be a consequence of the inability of both hematopoietic precursor and phagocytic cells to efficiently colonize and remodel this site (Fig. 3O, P). Collectively, the analyses of endothelial markers coupled with microangiography illustrate that knockdown of *pdc2* does not appear to cause a primary defect in vasculogenesis, and that patent vessels capable of serving as a conduit for

blood cell circulation are formed. Moreover, the *pdc2* morphant phenotype appears to be distinct from that of silent heart mutants with defects in cardiac development and blood flow, where large numbers of blood cells are released and pooled in major vessels [16].

Pdc2 has a cell autonomous function(s) required for erythroid maturation and HSC formation

The defects in the release of erythroid cells were mirrored by defects in the expression of erythrocyte-specific markers: *pdc2* morphants had reduced expression of hemoglobin alpha embryonic-1 marker (*hbae1*) (Fig. 4A, B), and lacked hemoglobin-stained erythrocytes as evidenced by the lack of DAF-stained cells at both 48 and 120 hpf (Fig. 4C–F). To study the morphological changes associated with loss of

FIG. 4. Impaired erythropoiesis and HSC development in *pcd2* morphants. (A, B) WISH of the erythroid marker *hbae1* in control (A) and 5ssMO-injected (B) 48 hpf embryos (arrow) (Control, $n=85/91$, 93%; 5ssMO, $n=64/66$, 97%). (C–F) Reduced DAF staining and hemoglobin containing erythrocytes in *pcd2* morphants compared to controls at 48 dpf (C vs. D) and 5 dpf (E vs. F). (G–J) Morphology of clusters of DsRed⁺ cells sorted from *pcd2* morphants and control embryos at 24 hpf (G, H) and 72 hpf (I, J). Polychromatophilic erythroblasts (b) from controls (G), or *pcd2* morphants (H) at 24 hpf, and Proerythrocytes (p), immature erythrocytes (i), and mature erythrocytes (e in insert) from controls (I) and *pcd2* morphants (J) are shown. (K) Average cell sizes, nuclear sizes (mean $\pm$ s.e.m), and nuclear/cytoplasmic ratio (N/C) of sorted DsRed⁺ cells at 24 hpf from control MO or *pcd2* *gata1:DsRed* morphants. (L) Mean erythroid DsRed⁺ cells ($\pm$ s.e.m) sorted from control and morphant embryos at 72 hpf. Data were determined in 3 independent experiments ($*P < 0.001$). (M) Top, schematic illustration of the blastula transplantation strategy used to investigate whether *pcd2* hematopoietic effects are cell-autonomous. Cells from control MO- or 5ssMO-injected CD41-EGFP transgenic donor embryos were transplanted at the blastula stage of either WT or 5ssMO-injected *gata1:DsRed* recipients. Donor HSCs contribution (arrows) at the PBI/CHT region was detected from control MO-injected cells in *gata1:DsRed* recipients, but none of 5ssMO-treated donor cells contributed to recipient HSC formation ($n=12$ embryos/transplant). Bottom, HSC chimerism determined according to percentage of embryos from either control MO- or 5ssMO-injected CD41-EGFP transplanted donor cells that contributed to either WT or *gata1:DsRed* recipient HSC formation in 3 independent experiments ($*P < 0.001$, Fisher's exact test). DAF, 2,7-diaminofluorene; dpf, days post-fertilization; HSC, hematopoietic stem cell; WT, wild type. Color images available online at www.liebertpub.com/scd

pcd2 function(s) in erythrocytes, we examined cytoplasmic preparations from sorted DsRed⁺ cells from *gata1:DsRed* *pcd2* morphants. Morphological examination revealed that *pcd2* morphant cells have significantly larger polychromatophilic proerythroblasts with open chromatin at 24 hpf (Fig. 4G, H). At 72 hpf, these cells frequently appeared as large multinucleated cells with highly condensed nuclear

chromatin and vacuolated cytoplasm that may represent apoptotic cells (arrows in Fig. 4J). Many of the *pcd2* morphant cells failed to differentiate into mature primitive erythrocytes (inset in Fig. 4J). Consistent with their classification as precursor erythroblasts, they were significantly larger in size, and had higher nuclear/cytoplasmic ratios compared to control cells (Fig. 4K). Additionally, at 72 hpf,

gata1:DsRed pdc2 morphants had increased numbers of polychromatophilic erythroblasts and proerythroblasts than controls (Fig. 4L). Together, these data suggest that embryos with reduced *pdc2* function accumulate hematopoietic precursors, and that *pdc2* is required for completion of the erythroid differentiation program.

To test whether *pdc2* effects on erythroid development are cell-autonomous, DsRed⁺ cells were sorted from mismatch- or 5ssMO-injected *gata1-DsRed* embryos at the 30-somite stage, and were transplanted into the dorsal aortic region of age-matched nonfluorescent wild-type hosts. From 20 recipient embryos transplanted each with 1–20 DsRed⁺ cells, 66% of cells from mismatch MO-treated embryos were released into the embryonic circulation, and contributed to the chimeric host erythropoiesis. In contrast, only 37% of transplanted cells from 5ssMO-treated embryos were released into the circulation, and the remaining cells accumulated at the yolk, and failed to contribute to host erythropoiesis (Fisher's exact test, $P < 0.001$, $n \geq 20$). This observation suggests that intrinsic functional defects within the erythroid lineage preclude the movements as well as the morphological and biochemical changes normally associated with erythroid differentiation.

We next sought to determine whether *pdc2*'s effects on HSC emergence were cell-autonomous. We utilized a *CD41:EGFP* line, which labels both thrombocytes and HSCs. To investigate whether thrombocytes and HSCs are formed in *pdc2* morphants, we injected the 5ssMO into the *CD41:EGFP* embryos. In this transgenic line, HSCs are GFP^{low}, and appear first in the AGM at 33 hpf, while thrombocytes are GFP^{high} and are detected first in the CHT at 48 hpf [30]. Consistent with the effects of *pdc2* knockdown on the HSC markers, *c-myb* and *runx1* (see below), *CD41:EGFP pdc2* morphants exhibited a failure to populate the AGM with any *CD41:EGFP*⁺ HSCs at 36 hpf (Supplementary Fig. S5A, B).

Transplantation of cells at the blastula/early gastrula boundary destined to form blood cell progenitors from *CD41-EGFP* embryos treated with a control MO into either WT or *gata1-DsRed* 5ssMO-treated embryos revealed that cells from control MO-treated embryos populate the PBI/CHT with donor-derived GFP^{low} HSCs or GFP^{high} thrombocytes in *pdc2* morphant recipient embryos (Supplementary Fig. S5C–E). However, blastula cells from *pdc2* morphants failed to populate recipient PBI/CHT with HSCs at 36 hpf or thrombocytes at 48 hpf either in WT or in *gata1:DsRed* recipients (Fig. 4M), therefore suggesting that *pdc2* effects on HSC emergence and/or the appearance of thrombocytes are cell-autonomous.

Pdc2 is required for terminal differentiation of multiple hematopoietic lineages

We used WISH to assess hematopoietic specification through the expression of transcription factors that constitute the regulatory transcriptional hierarchy in *pdc2* morphants. Knockdown of *pdc2* had no significant effect on the expression of either the paraxial mesoderm marker *myod* (Data not shown), the mesoderm marker *ntl* (Supplementary Fig. S6A, B), or the hematopoietic embryonic marker *cdx4* (Supplementary Fig. S6C, D), suggesting that loss of *pdc2* has no clear effects on mesodermal differentiation and patterning. We then examined the effects of *pdc2* knockdown on the expression of markers of the hemangioblast (*scl/tal1*), or HSCs (*runx1* and *c-myb*) (Fig. 5A, B). The expression of *scl* indicating hemangioblast formation [4] in both the ALM and PLM was stronger in *pdc2* morphants than in controls (Supplementary Fig. S6G). The upregulation of *scl* upon *pdc2* knockdown was further confirmed using quantitative real-time (RT)-PCR (Q-PCR) (Supplementary Fig. S6H). In contrast, *pdc2* morphants demonstrated a reduction in *c-myb* expression

FIG. 5. *Pdc2* knockdown modulates the expression of hematopoietic transcription factors. (A) WISH of *runx1* at the 10-somite stage (13.5 hpf) in WT, *pdc2* 5ssMO-injected embryos, or *pdc2* mRNA-injected embryos. Note the upregulation of *runx1* with mRNA injection (arrows). (B) WISH of *cmyb* and *runx1* at 30 hpf demonstrating loss of HSCs in *pdc2* morphants and upregulation of *runx1* transcript with the *pdc2* mRNA (*cmyb* control, $n = 111/113$, 98%; *cmyb* 5ssMO, $n = 165/180$, 92%; *runx1* control, $n = 117/123$, 95%; and *runx1* 5ssMO, $n = 84/93$, 90%). (C) WISH of *mpo*, and *l-plastin* at 30 hpf. (*mpo* control, $n = 40/42$, 95%; *mpo* 5ssMO, $n = 38/41$, 93%; *l-plastin* control, $n = 65/68$, 96%; and *l-plastin* 5ssMO, $n = 67/78$, 86%). The mean numbers ($\pm$ s.e.m) of *mpo*⁺ and *l-plastin*⁺ cells/embryo are significantly reduced in *pdc2* morphants (red bars) compared to WT (blue bars) (* $P < 0.001$). (D) WISH of lymphoid progenitor marker *rag1* at 96 hpf, and mature T-cell marker *lck* at 5 dpf, respectively (*rag1* control, $n = 28/38$, 74%; *rag1* 5ssMO, $n = 49/51$, 96%; *lck* control, $n = 53/56$, 95%; and *lck* 5ssMO, $n = 46/46$, 100%). Arrows point to the thymus. Scale bar, 500 μ m. mpo, myeloperoxidase. Color images available online at www.liebertpub.com/scd

(Fig. 5B), and a near complete loss of *runx1*-expressing cells in the ventral wall of the zebrafish dorsal aorta (Fig. 5B), suggesting failure of HSC initiation. On the contrary, the expression of *lmo2*, an early hematopoietic and vascular endothelial cell marker, appeared to be relatively normal in comparison to control embryos (Supplementary Fig. S6E, F). The relationship between *pdc2* and *runx1* became apparent from experiments increasing the dosage of *pdc2* through mRNA injection. Injection of 350 pg of *pdc2* mRNA at the 1-cell stage caused upregulation of *runx1* in the PLM at the 10 somite stage and throughout the embryo at 30 hpf (Fig. 5A, B). Thus, there appears to be a correlation between *pdc2* expression and *runx1* levels and/or Runx1 expressing cells. These data suggest that HSC emergence was disrupted by *pdc2* deficiency, and that this effect may be, at least in part, a consequence of *pdc2*'s effects on *runx1* expression.

The effects of *pdc2* on the expression of *scl*, *runx1*, and *c-myb* would likely result in changes in expression of their downstream targets and/or cell fate decisions. We assessed these changes using WISH with lineage-specific hematopoietic markers. It is important to note that the effects on markers of myeloid differentiation at 30 hpf might represent an aggregate effect of *pdc2* knockdown on both primitive and definitive hematopoiesis, with a likely contribution from short-term progenitors (EMPs) [5]. There was a significant reduction in hematopoietic cell maturation (Fig. 5C) with reduced expression of both myeloid-specific *myeloperoxidase* (*mpo*) (184 ± 12 *mpo*⁺ cells/control vs. 102 ± 8 *mpo*⁺ cells/*pdc2* morphant), and macrophage/myeloid-specific marker *l-plastin* (75 ± 8 *l-plastin*⁺ cells/control vs. 38 ± 11 *l-plastin*⁺ cells/*pdc2* morphant) indicating a failure of early embryonic myelopoiesis. We have demonstrated that *pdc2* morphants display a strong reduction in *hbae1* expression (Fig. 2C, D). Furthermore, the expression of erythroid and myeloid cells from both the primitive and definitive hematopoietic waves (*hbae1*, *mpo*, *plastin*) as well as definitive cell types, such as common lymphoid cell progenitors (*rag1*) and T-cells (*lck*), were markedly reduced in *pdc2* morphants (Fig. 5D).

Additionally, cells expressing the erythroid marker *gata1* failed to appear at the yolk and collected at the PBI/CHT (Supplementary Fig. S7A), while cells expressing the early myeloid progenitor marker *pu.1* were reduced at 30 hpf, and *pu.1*⁺ cells failed to appear in the PBI/CHT in *pdc2* morphants (Supplementary Fig. S7B). These data were supported by diminished Sudan Black staining of more mature neutrophils in the PBI/CHT of *pdc2* morphants at 48 hpf (Supplementary Fig. S7B) (73 ± 4 Sudan black⁺ cells/control embryo vs. 37 ± 5 Sudan black⁺ cells/*pdc2* morphant). *Pu.1* expression was partially rescued with the forced expression of the zebrafish *pdc2* mRNA, suggesting that *pdc2* morphant phenotypes were due to loss of *pdc2* function (Supplementary Fig. S7B). Collectively, these data support a model, where *pdc2* effects on bipotent EMPs [5] are essential for maintenance, but not the initiation of these progenitors, while loss of *pdc2* function(s) interferes with the initiation and/or maintenance of HSCs.

Loss of *pdc2* results in mitotic defects and ensuing P53-independent apoptosis

Given our observations with markers for hematopoietic development, we reasoned that the defect in hematopoietic cell maturation in *pdc2* morphants might be attributed to

aberrant progenitor maintenance and/or proliferation, apoptotic loss of differentiated cell types, or a combination of both of these processes. To help clarify the effects of *pdc2* loss, we first assessed the mitotic index of cells derived from morphant embryos using a mitosis-specific p-H3 antibody. We found a significant increase in the mitotic index in cells of the trunk and tail of the embryo proper at 24 hpf (Fig. 6A and Supplementary Fig. S8A–F). This difference could be attributed to an increase in S-phase entry/progression, or delayed mitotic exit. BrdU labeling demonstrated similar levels of S-phase occupancy between morpholino and control embryos at 30 hpf, and examination of DNA content of cells from controls and *pdc2* morphants by flow cytometry revealed no difference in S-phase progression (Supplementary Fig. S8I). Cells from control embryos showed typical distribution of normal cycling cells at 24 hpf (G1 = 64%, S = 31%, and G2/M 5%). In contrast, *pdc2* morphant cells had a significant increase in the sub-G1 population and a significant decrease in G1 subsets of cells consistent with cell death likely due to apoptosis (Fig. 6B). This effect extends out to 72 hpf, the latest time point tested. Consistent with this observation, we found that the number of TUNEL⁺ cells in *pdc2* morphants were significantly higher than controls at both 18 and 24 hpf ($n = 120$; effect seen in $95\% \pm 3\%$ of 5ssMO-injected embryos, $P < 0.001$) (Fig. 6C–G). The apoptotic effect observed appears to occur via a caspase-dependent cascade as quantification of activated caspase-3-positive cells in 30 hpf embryo trunks showed a 10-fold increase in the number present in morphant embryos relative to controls (Fig. 6F). While the apoptotic effect was clearly not restricted to regions of the embryo undergoing active hematopoiesis (e.g., PBI), we noted from examination of cytological preparations from TUNEL-labeled embryos that regions known to be active sites of hematopoiesis, such as the PBI, contained a larger number of apoptotic cells (Fig. 6C–E). Moreover, our cytospin preparations of *gata1:DsRed* erythroid cells identified cells that appeared apoptotic (Fig. 4J). Therefore, we attempted to determine whether the effect(s) of *pdc2* knockdown upon erythropoiesis might occur through an apoptotic mechanism. To this end, we sorted cells from *gata1:DsRed* embryos injected with *pdc2* and control morpholinos at 24 and 72 hpf, and determined the DNA content profile of DsRed⁺ erythrocytes. As suggested from the previous cytology, *pdc2* loss was associated with increased levels of apoptosis in the erythroid lineage with a large increase of sub-G1 cells in *pdc2* knockdown embryos relative to controls (Fig. 6G).

Given the absence of evidence for increased proliferation, we deduced that the increase in mitotic index observed in *pdc2* morphants might be a function of failed mitoses or a delay in mitotic exit. Therefore, we stained *pdc2* morphant embryos with antibodies to p-H3 and β -tubulin to determine if the spindle structure was affected. Interestingly, *pdc2* morphants often displayed defective spindle formation most likely derived from an inability to properly position chromosomes at the metaphase plate (19% *pdc2* morphant vs. 3% control; $P < 0.005$; Fig. 6H). The presence of monotelic chromosome attachment is predictive of mitotic delay by virtue of the spindle assembly checkpoint [41], and the degree to which such defective figures are present in *pdc2* morphant embryos provides a likely explanation for the increase in mitotic index.

Since *p53* is involved in the regulation of apoptosis in hematopoietic progenitors, and extensive cell death could

FIG. 6. Mitotic defects in *pdc2* morphants are due to defective spindle assembly. **(A)** Quantification of IHC mitotic cells in control and *pdc2* knockdown embryos using p-H3 antibodies (see representative images; Supplementary Fig. S8A–F). Mitotic index calculated as p-H3⁺ cells over DAPI⁺ cells in control and *pdc2* morphants at 24 hpf. (**P* < 0.05). **(B)** Summary of propidium iodide DNA content profiles for both control and *pdc2* knockdown (5ssMO) as determined by flow cytometry or whole dissociated embryos (for a representative profile, see Supplementary Fig. S8I). *Pdc2* morphants have significantly fewer cells in G1 and increase in the number of sub-G1 cells presumed to be apoptotic based on further analyses. *Indicates an increase, **indicates a decrease compared to control. *, ***P* < 0.01. **(C, D)** Whole mount TUNEL labeling of 48 hpf embryos. Dashed boxes highlight the region encompassing the CHT that is actively undergoing hematopoiesis at this time point. **(E)** Scoring of the mean number of TUNEL⁺ cells in 48 hpf embryos (**P* < 0.001). **(F)** Scoring of the mean number of activated caspase⁺ cells in 48 hpf embryos (**P* < 0.001). **(G)** Cell cycle stage analysis (24 and 72 hpf) based on DNA content profiles for sorted *gata1:DsRed* positive cells labeled with DAPI. Control and *Pdc2* knockdown *gata1:DsRed* embryos were dissociated and labeled with DAPI and subjected to fluorescent activated cell sorting analysis to determine cell cycle stage. At both 24 and 72 hpf, there was an increase in apoptotic erythroid cells (*gata1*⁺). **(H)** Immunofluorescence images of asynchronous cells from control, and 5ssMO-injected 26 hpf embryos stained with b-tubulin (red), p-H3 (green), and DAPI (blue). Lower panels are magnifications of the outlined area. Note the 2 examples of chromosomes with apparent monotelic attachment in cells from *pdc2* morphants. Scale bars are 100 μm in (C, D) and 10 μm in (H). p-H3, phospho-histone H3. Color images available online at www.liebertpub.com/scd

occur due to *p53* activation as an off-target effect of morpholino injection [42], we investigated a possible role for *p53* in the hematopoietic defects seen in *pdc2* morphants. We coinjected the *pdc2* and *p53* MOs, and we still observed a significant reduction in the numbers of *runx1*⁺ cells in the ICM at 30 hpf (Supplementary Fig. S9D) that could be restored by coinjection of the *pdc2* RNA (data not shown, and Supplementary Fig. S9E). These data suggest that *pdc2* effects are specific, and that *p53* loss cannot fully restore the impairment of HSC formation. This was further confirmed by knockdown of *pdc2* in *p53* homozygous mutant embryos. Injection of *pdc2* ATGMO or 5ssMO in *p53*^{-/-} embryos led to impaired release of erythrocytes into the circulation, and a significant reduction in *runx1* expressing cells in the ICM at 30 hpf similar to that observed in *p53*^{wt/wt} embryos (Data not shown) further substantiating the evidence that *pdc2* effects on *runx1* expressing HSCs and erythroid maturation are specific and largely *p53*-independent.

Restoration of *runx1* expression and inhibition of apoptosis reverse the effects of *pdc2* loss on erythropoiesis

Jak2/Stat signaling transduces signals of hematopoietic cytokines to enhance erythroid cell proliferation. Treatment of zebrafish embryos with the *jak* inhibitor AG490 reduced phosphorylated *stat5* [43]. Reduction in phosphorylated *Stat5* might result in upregulation of gene expression of *Stat5*-target genes that include *runx1*. Moreover, *STAT5* co-immunoprecipitates with *RUNX1*, and inhibits *RUNX1* nuclear localization and transcriptional activity [44]. Thus, we investigated if pharmacological inhibition of *stat5* activity might restore *runx1* function(s) in *pdc2* morphants. We first determined whether AG490 treatment resulted in increased *runx1* transcript levels in 30 hpf embryos. Examination of *runx1* transcripts by WISH supports this observation with the increase being most visible in the anterior aspect of the AGM (inset AG490, Fig. 7A). We then asked whether treatment with AG490 would de-repress the attenuated expression of *runx1* observed in *pdc2* knockdown embryos. First, we examined the expression of *runx1* by WISH and observed that *runx1* transcripts are present throughout the AGM in *pdc2* morphant embryos treated with AG490 (Fig. 7A). We dissected trunks and tails of embryos to minimize the contribution of neurally expressed *runx1* and examined *runx1* transcript levels. Q-PCR analyses revealed a significant increase in *runx1* expression in dissected trunks of AG490-treated morphant embryos (Fig. 7B). These data suggest that *pdc2* effects possibly involve *runx1*-mediated maintenance of HSCs.

We then attempted to determine whether the effects of *pdc2* knockdown on erythroid differentiation were modulated by de-repression of *runx1*. We utilized DAF reactivity as a measure for the presence of mature hemoglobinated erythrocytes. Treatment of control embryos with 50 μ M AG490 resulted in no apparent effect on the number of DAF⁺ erythrocytes at either 48 or 96 hpf. However, similar treatment of *pdc2* morphant embryos revealed a dramatic increase in the number of mature erythrocytes relative to vehicle-treated embryos. These results suggest that the de-repression of *runx1* afforded by the inhibition of *Jak/Stat* activity relieves *pdc2*'s effects on erythroid differentiation block.

The ability of *jak/stat* inhibition to rescue the effects of *pdc2* knockdown may be attributed to downregulation of *scl* and/or other progenitor genes, modulation of apoptosis, in addition to increased *runx1* expression after AG490 treatment (Fig. 7A, B). An increase in *scl* expression has been previously reported for embryos with reduced *runx1* function [12], we found similar effects on *scl* expression in *pdc2* morphants that could be reversed by injection of the *pdc2* mRNA (Fig. S6H). Previous analysis of *runx1* morphants indicated defects both in definitive HSC formation, and an accumulation of immature hematopoietic progenitors coincident with a block to erythroid differentiation [12]. We reasoned that *Jak/Stat* inhibition might also have distinct effects on modulating apoptosis. Therefore, we examined apoptosis through TUNEL labeling in both control and *pdc2* morphant embryos in the presence of the *Jak* inhibitor. We assessed apoptosis in 30 hpf embryos within a region encompassing the AGM, including the posterior blood island. The analysis of these embryos indicated a significant decrease in the number of apoptotic cells in *pdc2* morphant embryos when treated with *Jak* inhibitor versus vehicle controls, suggesting that inhibition of apoptosis is, at least in part, responsible for the rescue observed in AG490-treated embryos (Fig. 7D). Notably, in the absence of *pdc2* knockdown, AG490 treatment caused a slight, but not a significant increase in TUNEL-positive cells in the AGM/PBI region of control embryos. Thus, we conclude that loss of *pdc2* function(s) during zebrafish hematopoietic development results in erythroid differentiation arrest, likely at the proerythroblast stages, and failure of HSC emergence. These effects could be attributed to modulated *runx1* expression and/or function (s), mitotic defects, and *p53*-independent caspase-dependent apoptosis.

Discussion

Hematopoietic progenitors have to rapidly integrate extrinsic and intrinsic cues that activate or repress transcription factors controlling cell fate. *Pdc2* knockdown led to ineffective hematopoiesis, pooling of hematopoietic progenitor cells that failed to release into circulation over the yolk, impaired hematopoietic cell differentiation, mitotic defects, and apoptosis. The resulting pancytopenia was associated with upregulation of *scl* during primitive hematopoiesis, and downregulation of the HSC markers, *runx1* and *cmyb*. These results are consistent with requirement of *pdc2* function(s) for maintenance, but not for initiation of primitive hematopoietic progenitors. Moreover, loss of *pdc2* function(s) interferes with initiation and/or maintenance of HSCs. There are several possible mechanisms for the *pdc2* morphant phenotype. One is that reduced *pdc2* levels in the ICM affected hematopoietic differentiation through regulating the expression of *runx1*, *scl*, their downstream targets, soluble factor(s), or other essential hematopoietic gene(s) that were not examined in this study. *Scl* is essential for EMP lineage commitment, but not for HSC maintenance in the adult human BM [45]. In mouse studies, *Scl* and *Runx1* were shown to be necessary for the initiation, but dispensable for the maintenance of HSCs [46]. The lack of hematopoietic clusters in the AGM of *Runx1*^{-/-} mice suggests that *Runx1* regulates hematopoietic endothelial cell transition in the dorsal aorta. Indeed, knockdown of *runx1* by morpholino in zebrafish

FIG. 7. Upregulation of *runx1* and modulation of apoptosis through inhibition of Jak reverses the effects of *pdcd2* knock-down on erythropoiesis. **(A)** WISH of *runx1* expressing cells in the arterial and AGM regions of treated embryos at 30 hpf. Treatment with AG490 resulted in increased *runx1* expression in the arterial and AGM regions (upward arrowheads in the AG490 panel), and possibly expansion of the anterior myelopoietic compartment (downward arrowheads). Note the dramatic rescue of *runx1* expression in the ICM with the combination of AG490 and 5ssMO compared to the combination of vehicle and 5ssMO on top. (Vehicle $n=35$ (71%); AG490 $n=28$ (86%); 5ssMO $n=34$ (91%); and 5ssMO⁺ AG490 $n=36$ (14%) showed the displayed phenotypes). **(B)** Q-PCR analyses of *runx1* expression relative to β -actin in the AGM region of *pdcd2* morphants after AG490 treatment (mean $\pm$ s.e.m., * $P<0.05$). Scale bar, 500 μ m. **(C)** DAF staining of hemoglobinated cells in WT, or 5ssMO-injected embryos at 48, or 96 hpf treated with either vehicle, 50 μ M AG490, *pdcd2* 5ssMO, or combinations of 5ssMO and AG490. Numbers and percentages of embryos representing the demonstrated DAF⁺ staining are for WT with vehicle at either 48 or 96 hpf, $n=23$ (100%), WT with AG490 at 48 hpf, $n=18$ (100%), and at 96 hpf, $n=31$ (100%). For 5ssMO treated with vehicle at 48 hpf, $n=20$ (40%), and at 96 hpf, $n=24$ (21%), and 5ssMO treated with AG490 at 48 hpf, $n=22$ (68%), and at 96 hpf, $n=14$ (86%). The effects of combined 5ssMO⁺ AG490 treatments were significant when compared to individual treatment ($P<0.01$). **(D)** Left, representative images from the 4 groups of TUNEL-stained 30-hpf embryos. Insets demonstrate the AGM/PBI region where hematopoietic cells were counted. Right, quantification of the antiapoptotic effects of Jak inhibition in *pdcd2* morphant embryos. Sixteen embryos were analyzed for each treatment from the AGM/PBI region highlighted in **(A)** in 3 experiments with 3 investigators blinded to treatment performing the counts for TUNEL-positive cells. Data for each embryo are derived from a series of Z sections spanning the depth of the embryo, and then numbers of apoptotic cells were averaged from counts of 3 investigators. (* $P<0.01$; ** $P<10^{-4}$; bars s.e.m.). Scale bar, 500 μ m. Color images available online at www.liebertpub.com/scd

decreases the hematopoietic endothelial transition and induces apoptosis in these cells [8]. Therefore, failure of progenitor cell circulatory release in *pdcd2* morphants may occur due to prolongation of an uncommitted progenitor state from persistent *scl* expression, or due to loss of *runx1* expression in the ICM. These 2 possibilities are interdependent as *runx1* knockdown embryos exhibited persistent *scl* expression in zebrafish [12]. Mouse yolk sac erythrocyte progenitors were shown to clump at the yolk, while other cells circulate freely [38], suggesting a change of adhesive properties of primitive blood cells as they are released to the circulation. Similarly, zebrafish blood cells were recently shown to detach from the endothelial wall by cell-autonomous proteolytic degradation of extracellular adhesion molecules [36]. These coordinated events would likely require that blood cells be released into circulation only when they reach a specific differentiation state, or that an inhibitory mechanism to hematopoietic cell intravasation is reversed. Whether *pdcd2* function(s) is involved in this regulatory process remains to be investigated.

Transplantation studies suggest that *pdcd2* acts predominantly in a cell autonomous fashion to initiate and maintain a definitive HSC pool in the CHT/PBI. Moreover, *pdcd2* deficiency leads to abnormal HSC differentiation and apoptosis that occurs mostly independent of direct *p53* activity. Studies in rat embryonic fibroblasts determined that induction of the *pdcd2* homologue Rp8 is independent of *p53*-mediated apoptosis [47]. Flies with mutations in the *pdcd2* homologue, *zfrp8*, displayed lymph gland hyperplasia with defects in hemocyte differentiation. Hyperplasia of hemocytes that are confined within the drosophila lymph gland appears similar to the accumulation of cells over the yolk of zebrafish *pdcd2* morphants. However, distinct signaling pathways and stages of differentiation during the multiple waves of hematopoiesis in vertebrates might explain the differences between mutant fly and zebrafish phenotypes. Circulating cells during fly larval hematopoiesis are mostly plasmatocytes that are destined to become macrophage-like cells, and there are no cellular equivalents to red blood cells or lymphocytes [48]. The posterior signaling center (PSC) in fly lymph gland maintains blood precursors under control of signals, such as the *runx1*-homologue *Lozenge* [49]. Regulation of HSCs in *zfrp8* mutants is dependent on these PSC signals [22]. Since zebrafish *pdcd2* morphants showed increased expression of *scl* and decreased expression of *runx1*, it appears that a conserved cross talk between *pdcd2* and *scl* and/or *runx1* might regulate HSC maturation.

Jak/stat signaling is activated during intermediate stages of lineage commitment of developing zebrafish erythroid progenitors [50]. Erythropoietin (epo)-mediated activation of *stat5*, *PI3K* signaling, and *bcl-xl* response leads to modulation of apoptosis [51] through regulation of STAT-target genes encoding apoptosis inhibitors (e.g., Bcl-xL, Mcl-1, and survivin) and cell cycle regulators (e.g., cyclins D1/D2 and c-Myc) [52]. Loss of zebrafish *stat5* blocked the expansion of erythrocytes by exogenous *epo*, demonstrating a conserved role for *stat5* in zebrafish erythropoiesis [53]. Treatment with AG490, a soluble *jak2* inhibitor, reduced phosphorylated *stat5* [43], which is essential for *bcl-xl* induction and survival [54], and restored *runx1* expression in rescued *pdcd2* morphants. While inhibition of STAT3/5 signaling almost al-

ways results in growth inhibition and induction of apoptosis in tumor cells, disruption of Stat3 signaling in normal fibroblasts causes growth arrest, but not apoptosis [55,56], suggesting that normal cells, unlike tumor cells, might not depend on stat signaling to sustain their survival. Thus, restoration of *runx1* expression, and inhibition of *stat5/bcl-xl*-mediated apoptotic pathways during primitive erythropoiesis are 2 potential mechanisms by which the effects of *pdcd2* were reversed with AG490 treatment. It will be critical to identify *pdcd2* interacting partners and determine the mechanisms of cross talk between *pdcd2*, *runx1*, and *Jak/Stat* signaling.

Hematopoiesis migrates successively from the yolk sac and fetal liver or placenta in mammals, or from CHT/PBI in zebrafish, to the bone or kidney marrow. The properties of yolk or CHT/PBI and marrow HSCs reflect diverse environments and intrinsic HSC features at each stage. For instance, fetal liver HSCs have active cell cycle, whereas adult marrow HSCs are largely quiescent. *Pdcd2* knockdown in embryos induced mitotic defects with a higher mitotic index. The spindle assembly checkpoint guards against chromosome mis-segregation by delaying cell cycle progression through mitosis, and defects in the mitotic checkpoint generate aneuploidy and might facilitate tumorigenesis [57]. Cells from *pdcd2* morphants appeared to progress through the cell cycle, although with an aberrant mitosis and with frequent monotelic chromosome orientation. Progression from metaphase to anaphase with monotelic orientation produces chronically activated checkpoints that provoke cell death and subsequent apoptosis through an undefined pathway [57]. Whether similar mitotic defects played a role in the inviability of *pdcd2* ESCs in mice [25] will be important to investigate.

Deregulated *pdcd2* expression has been implicated in human leukemias and lymphomas [27]. Our studies suggest that *pdcd2* maintains progenitor cells through interactions with *runx1* and other cell fate-determining factors. Clearly, further studies will be necessary to more precisely determine the functions of *pdcd2* during hematopoietic cell differentiation and survival. Loss of *pdcd2* function(s) in zebrafish perturbs HSC differentiation due to mitotic defects during cell cycle progression and *p53*-independent apoptosis. These observations, the findings that *PDCD2* is strongly expressed in adult hematopoietic tissues, and our recent study demonstrating the requirement of human *PDCD2* in erythroid progenitor cell differentiation [58] suggest that *PDCD2* controls transcriptional networks regulating hematopoietic and other progenitor cell development, and therefore would likely participate widely in the oncogenic process. The challenge for future studies will be to elucidate the basic mechanisms of *pdcd2* function, including the identification of direct targets and partners that allow *pdcd2* to regulate HSC fate, to determine how *pdcd2* repression or activation is differentially achieved in different cells, and to understand how *pdcd2* might participate in tumor development.

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Author Disclosure Statement

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