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Retinoic Acid Signaling Is Essential for Embryonic Hematopoietic Stem Cell Development

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SUMMARY

Hematopoietic stem cells (HSCs) develop from a specialized subpopulation of endothelial cells known as hemogenic endothelium (HE). Although the HE origin of HSCs is now well established in different species, the signaling pathways that control this transition remain poorly understood. Here, we show that activation of retinoic acid (RA) signaling in aorta-gonad-mesonephros-derived HE ex vivo dramatically enhanced its HSC potential, whereas conditional inactivation of the RA metabolizing enzyme retinal dehydrogenase 2 in VE-cadherin expressing endothelial cells in vivo abrogated HSC development. Wnt signaling completely blocked the HSC inductive effects of RA modulators, whereas inhibition of the pathway promoted the development of HSCs in the absence of RA signaling. Collectively, these findings position RA and Wnt signaling as key regulators of HSC development and in doing so provide molecular insights that will aid in developing strategies for their generation from pluripotent stem cells.

INTRODUCTION

In the mouse embryo, hematopoietic stem cells (HSCs) are specified in the aorta-gonad-mesonephros (AGM) between embryonic day (E) 10.5 and 11.5 through a sequence of developmental events that involves the transition of a specialized endothelial population known as hemogenic endothelium (HE)/pre-HSCs to hematopoietic progenitors, a process commonly referred as the endothelial to hematopoietic transition (EHT) (Medvinsky and Dzierzak, 1996; Yoshimoto and Yoder, 2009). During this transition, the developing hematopoietic cells undergo morphological changes, "bud" from the HE, and form distinct clusters that can be detected within the lumen of the aorta (Bertrand et al., 2010; Boisset et al., 2010; Eilken et al., 2009). The HE and early hematopoietic progeny share the expression of many endothelial and hematopoietic markers and transcription factors, including VEC, AA4.1, CD31, *Runx1*, *Gata2*, and *Scl/Tal1* (Bertrand et al., 2005; Chen et al., 2009b; Lancrin et al., 2009; Ren et al., 2010; Schlaeger et al., 2005; Taoudi et al., 2008; Tsai et al., 1994; Yokomizo et al., 2001) These populations can, however, be distinguished by expression of the pan hematopoietic marker CD45 because its levels are upregulated with the transition to a hematopoietic fate. Given these differences, emerging HSCs can be identified and isolated from the AGM of appropriately staged embryos based on the coexpression of VEC/AA4.1 and CD45 (Bertrand et al., 2005; Lancrin et al., 2009; Taoudi et al., 2008; Taoudi et al., 2005). Once formed in the AGM, it is thought that the HSCs seed the fetal liver where the population expands prior to moving to the bone marrow late in fetal life.

Expression analyses and gene targeting studies have identified key transcription factors that play a role in HSC development and in doing so have begun to define the transcriptional regulatory network that controls this developmental progression (Zon, 2008). The findings from these studies position *Runx1* as a central player in this process because it is expressed within the HE and newly formed hematopoietic cells and it is essential for the EHT and consequently for HSC development (Chen et al., 2009b; Mukouyama et al., 2000; Wang et al., 1996; Yokomizo et al., 2001). Additionally, *Gata2* and *Gata3*, *Scl/Tal1*, members of the *Hox* family, and *Sox17* are also expressed to varying degrees in the early vasculature, the HE, and early hematopoietic progenitors and have been shown to play a role in the development of functional HSCs (Antonchuk et al., 2002; Clarke et al., 2013; Fitch et al., 2012; Mikkola et al., 2003; Tsai et al., 1994). In contrast to our understanding of transcriptional regulation of hematopoietic development, the signaling pathways that control these early events are not well defined. Components of different pathways including Notch, Wnt, IL-3, Hedgehog, and BMPs are expressed in the region of the aorta that gives rise to HE and hematopoietic progenitors, suggesting they play some role in this process (Kaimakis et al., 2013). Of these, the Notch and Wnt pathways have been shown to be required for HSC development in vivo (Hadland et al., 2004; Kumano et al., 2003; Ruiz-Herguido et al., 2012).

In addition to these regulators, retinoic acid (RA) signaling is of interest in the context of embryonic hematopoiesis as it is known to function in many different lineages at multiple stages

of development and recent studies have shown that activation of the pathway can impact hematopoietic development in zebrafish and embryonic stem cell differentiation cultures (de Jong et al., 2010; Yu et al., 2010). RA signaling is mediated through one of the three retinoic acid receptors (RAR) -α, -β, and -γ that form hetero-dimerized complexes with the rexinoid receptors (RXR) -α, -β, and -γ (Rhinn and Dollé, 2012). Signaling is initiated following binding of the ligand all-trans retinoic acid (ATRA), a derivative of Vitamin A, and results in activation of downstream target genes (Balmer and Blomhoff, 2002, 2005; Duester, 2008). As ATRA acts at short distances, it is synthesized at the site of action, often in the responding cells, by one of the three different retinal dehydrogenases (Raldh-1, -2 and -3)—a subclass of aldehyde dehydrogenases (Duester, 2008; Kumar et al., 2012).

Both the RARs and the Raldhs are widely expressed throughout embryonic development, including the hematopoietic tissues (Duester, 2008; Kumar et al., 2012). Despite these patterns, targeting studies failed to uncover any gross hematological defects in *RAR-α*, *RAR-γ*, *Raldh1*, and *Raldh3* null embryos, suggesting that either the pathway is not essential for embryonic hematopoietic development or that compensatory mechanisms masked the function of these individual receptors and enzymes. *Raldh2* null embryos, in contrast, show hematovascular defects in the yolk sac (YS) at E9.5, suggesting that the pathway does play some role in hematopoietic development (Goldie et al., 2008; Niederreither et al., 1999). Detailed analyses of AGM hematopoiesis or HSC development was not, however, possible in these embryos as they die between E9.0 and E10.5.

In this study we investigated the role of RA signaling in hematopoietic development in the AGM and demonstrate that this pathway is required for the generation of HSCs. We show that activation of the pathway in isolated HE/pre-HSC populations in vitro dramatically enhanced the generation of HSCs, whereas conditional deletion of *Raldh2* in VEC+ endothelial cells in vivo inhibited HSC development. We also provide evidence that RAR-α is the effective RA pathway agonist for enhancement of HSCs and that the effects are mediated through the Wnt/β-catenin pathway. Together, these findings identify a pivotal role for RA signaling in HSC development.

RESULTS

Expression of RARs and Raldhs in AGM-Derived Hemogenic and Hematopoietic Populations

To determine whether RA signaling plays a role in embryonic HSC development, we analyzed E10.5 and E11.5 AGM-derived AA4.1+/VEC+ (E+) populations, known to contain HE (Bertrand et al., 2005; Rybtsov et al., 2011) and HSCs, respectively (Bertrand et al., 2005; Taoudi et al., 2008), for expression of retinoic acid receptors (RAR) -α, -β, and -γ that are functionally required to mediate RA signaling (Rhinn and Dollé, 2012). Intracellular flow cytometric analyses revealed that RAR-α and RAR-γ were expressed in the majority of the E+ cells at both time points, whereas expression of RAR-β appeared to be restricted to only a subset of this population at E11.5 (Figure 1A available online). All RARs were also expressed in the AA4.1−/VEC− population (data not shown). Immunostaining analyses further confirmed that RAR-α and RAR-γ were expressed in the dorsal aorta region at E11.5 (Figure 1B). The observation that RARs are expressed in these AGM populations supports the interpretation that RA signaling could play a role in HSC development.

The presence of retinal dehydrogenases (Raldhs), the enzymes responsible for synthesizing RA is also an indication of active RA signaling, as in many cases the cells that respond to it also synthesize it (Duester, 1999, 2008). To determine whether AGM-derived cells, including HSCs, display the capacity to synthesize RA, the E+ fraction from E10.5 and E11.5 AGMs was stained with aldefluor (Jones et al., 1995), a fluorescent compound that measures the enzymatic activity of active aldehyde dehydrogenases including retinal dehydrogenases (Kumar et al., 2012). As shown in Figure 1C, aldefluor + (Aldh+) populations were detected within the E+ fraction of the AGM at both time points, indicating that at least a subset of these cells expresses functional aldehyde dehydrogenase(s). To further characterize the enzyme activity in these cells, Aldh+ and Aldh− fractions from the E11.5 AGM-derived E+ population were isolated by FACS and analyzed for expression of 15 aldehyde dehydrogenases. As shown in Figure 1D, expression of *Raldh 1*, *2*, and *3* segregated predominantly to the Aldh+ fraction, whereas all other *Aldhs*, with the exception of *3B2*, were expressed at equal levels in both fractions. These findings suggest that the E+ population in the developing AGM has functional retinal dehydrogenases and as such, further support the notion that the RA signaling pathway is active in this region of the embryo at this stage of development.

Aldefluor Activity Tracks with HSC Potential in the AGM

Previous studies have demonstrated that developing HSCs can be isolated based on the coexpression of AA4.1+/VEC+ (E+) and CD45+ (H^+) (Taoudi et al., 2008). To determine if the developing HSCs display aldehyde dehydrogenase activity, the AA4.1+/VEC+CD45+ (E+H+) AGM fraction from E11.5 embryos was stained with aldefluor, and the resulting Aldh+ and Aldh− populations were isolated by FACS and transplanted into irradiated $Rag2^{-/-}\gamma c^{-/-}$ recipients (Figure 2A). At 16 weeks posttransplantation, donor-derived hematopoietic cells were detected only in mice transplanted with the E+H+Aldh+ population ($p < 0.01$) indicating that it contains all the HSC activity (Figures 2B and 2C). Donor-derived lymphoid and myeloid cells were detected in all primary hematopoietic organs in representative mice at 32 weeks posttransplantation, demonstrating multilineage repopulation (Figure S1A). Bone marrow from the 32-week-old recipients was able to repopulate secondary recipients, indicating the presence of long-term repopulating HSCs (Figure S1B). LDA revealed a HSC frequency of 1 in 0.78 embryo equivalent (e.e.) in the E+H+Aldh+ population (Tables S1 and S3). In contrast to HSCs, progenitors as measured by colony formation in methylcellulose, were detected in both populations. Interestingly, higher levels of multi-and bipotential (Multilineage, GM) progenitors were present in the Aldh− population (Figure 2D). Together, these findings show that, at this stage, the AGM-derived HSCs are Aldh+, whereas the majority of the progenitors segregate to the Aldh− fraction.

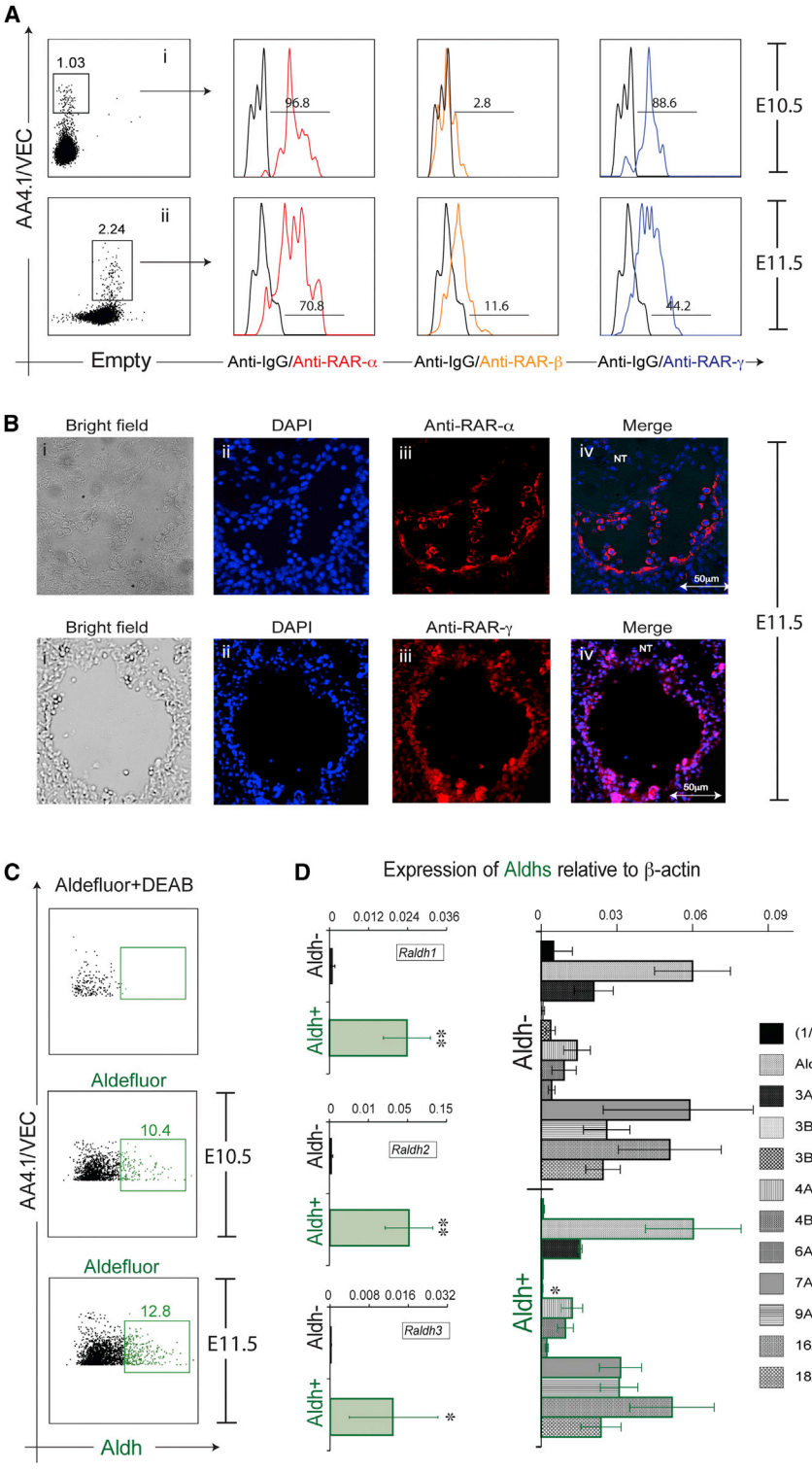

Figure 1. Retinoic Acid Receptors and Retinal Dehydrogenases Are Expressed in AGM-Derived HSC-Containing Populations

(A) Intracellular flow cytometry analysis of RAR (-α, -β, and -γ) in AA4.1+/VEC+ cells from the (i) E10.5 and (ii) E115 AGM region.

(B) Immunohistochemistry of the dorsal-aorta region (E11.5) stained with anti-RAR-α and RAR-γ antibodies.

(C) Aldefluor staining in AA4.1+/VEC+ cells from E10.5 and E11.5 AGM.

(D) qRT-PCR analyses of aldehyde dehydrogenases, implicated in RA or non-RA synthesis, in Aldh+ and Aldh− cells sorted from the E11.5 AGM-derived AA4.1+/VEC+ population. Significant differences are indicated with asterisks, and error bars represent the SEM (*$p < 0.05$, **$p < 0.01$, t test).

HSCs to E11.5 E+H+Aldh− hematopoietic progenitors (Figure 3A). qRT-PCR-based expression analyses of these populations revealed that genes associated with endothelial development, the specification of hemogenic endothelium (*Notch*), and the establishment of definitive hematopoiesis (*Hox*, *Scl*, *Aml*) were expressed at higher levels in the E10.5 populations, and in the HSC containing fraction (E+H+Aldh+) than in the progenitor fraction (E+H+Aldh−). In contrast, genes associated with the development of the hematopoietic lineages such as *Gata1*, *Gata2*, and the hematopoietic specific genes *CD41* and *CD45* were expressed at higher levels in the E+H+Aldh− fraction. Importantly, the retinal dehydrogenases *Aldh1a1* (*Raldh1*) and *Aldh1a2* (*Raldh2*) were the only genes whose expression tracked with the HSC containing populations (Figure 3A).

To test whether or not Raldh activity plays a role in HSC development, we deleted *Raldh2* in *VEC*+ cells, by crossing *VEC*-Cre and *Raldh2*-floxed mice as previous studies showed that *Raldh2* null mice display yolk sac hematopoietic defects. The AGM, yolk sac (YS) and placenta (PL) from *Raldh2*-deleted (*Raldh2*$^{f/f}$;*VEC*-Cre) and -nondeleted (*Raldh2*$^{f/+}$;*VEC*-Cre, *Raldh2*$^{f/+}$, *Raldh2*$^{f/f}$: controls) embryos were assayed for HSC potential (Figure 3B). As shown in Figures 3C and 3D, none of the recipients of the *Raldh2*$^{f/f}$;*VEC*-Cre AGM cells showed any repopulation indicating that this enzyme is essential for the generation of the HSC at this site. Two recipients from each of the YS and PL groups did contain

Raldh2 Is Required for HSCs Formation

Access to the AGM subpopulations at different stages enables one to establish a developmental progression transitioning from E10.5 (E+Aldh− and E+Aldh+) HE to E11.5 E+H+Aldh+

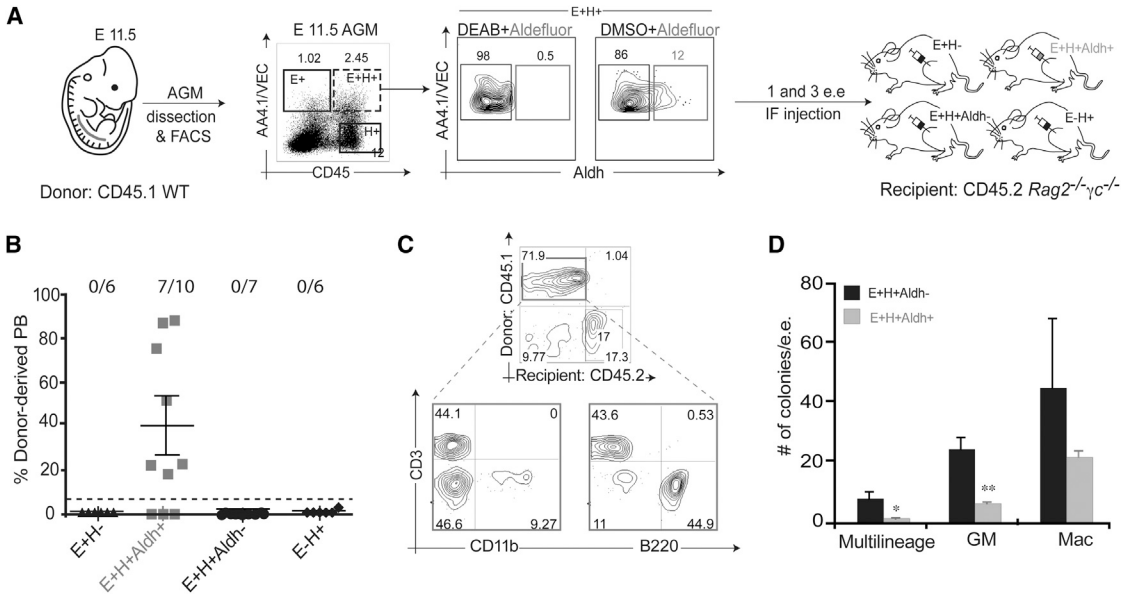

Figure 2. Aldehyde Dehydrogenase Activity Tracks with Emerging HSCs from the AGM

(A) Scheme for transplantation assay. E+H−: AA4.1+/VEC+CD45−; E+H+: AA4.1+/VEC+CD45+ (black dashed rectangle); E−H+: AA4.1+/VEC-CD45+. The aldefluor (Aldh) positive and negative fraction within E+H+ are identified as E+H+Aldh+ (gray solid rectangle) and E+H+Aldh− (black solid rectangle), respectively. Negative control, DEAB+Aldefluor, represents cells treated with the aldehyde dehydrogenase inhibitor DEAB.

(B) Engraftment potential of the E+H− (solid triangle), E+H+Aldh+ (solid rectangle), E+H+Aldh− (solid circle), and E−H+ (solid diamond) fractions at 1 embryo equivalent (e.e.) Significant difference from these combined fractions are indicated with asterisks (**$p < 0.01$, ANOVA and Dunnett's postmultiple comparison test). The dashed line represents 5% threshold level of donor-derived reconstitution, and error bars represent the SEM.

(C) Flow cytometric analysis to monitor donor-derived multilineage reconstitution in mice transplanted with E+H+Aldh+ cells.

(D) Methylcellulose-based colony assays measuring the progenitor potential of the E+H+Aldh− (black solid bars) and E+H+Aldh+ (gray solid bars) populations (*$p < 0.05$, t test). See also Figure S1 and Tables S1 and S3.

significant numbers of donor cells, possibly reflecting incomplete deletion of the *Raldh2* alleles. To address this, hematopoietic colonies generated from CD45.2 sorted (donor) cells from these recipients were analyzed by PCR (genomic) for the presence of the *Raldh2* alleles. The outcome of this analysis revealed that all of the colonies assayed (~200) contained a nondeleted allele, demonstrating that *Raldh2* is required for HSC development (Figure 3F).

Raldh2$^{f/f}$*;VEC*-Cre embryos showed subtle anatomical abnormalities, including defective development of the head, but were viable at E11.5, the time of HSC analyses, (Figure 3E). We did not observe any striking defects in the dorsal aorta of these embryos, suggesting the observed defect in repopulation frequency was not due to perturbed endothelium (Figure S2A). Together, these data demonstrate that *Raldh2* is required for HSC formation and/or function during embryogenesis.

RA Signaling via RAR-α Promotes HSC Development

To further investigate the role of RA signaling in HSC development, the E11.5 AGM-derived VEC+/AA4.1+ (E+) population was isolated by FACS, reaggregated and cultured for 8 hr in a serum-free medium with either ATRA, a pan-RAR agonist, or the RAR-α (AM580; Delescluse et al., 1991; Gianni et al., 1993), -β (AC55649; Lund et al., 2005) or -γ (CD437; Gianni et al., 1993) receptor-specific agonists (Figure 4A). Following this culture step, the cells were transplanted into recipient mice at 1 e.e. to determine if activation of the pathway influenced their repopulation potential (Figure 4A). The entire AA4.1+/VEC+ (E+) fraction containing both the pre-HSC endothelial (AA4.1+/VEC+CD45−) and HSC (AA4.1+/VEC+ CD45+) populations were used for these studies to maximize the potential progenitor pool that could respond to these agonists. Culture with ATRA resulted in a modest enhancement ($p = 0.0501$) in the proportion (mean [m] = 15%) of donor cells and the frequency (f = 60%) of repopulated recipients compared to the DMSO (m = 3.5%, f = 20%) control at both 16 (Figure 4B) and 32 weeks (data not shown) posttransplant. Treatment with AM580 in contrast led to a significant ($p < 0.01$) increase in both the proportion of donor cells (m = 40%) and frequency (f = 100%) of recipients engrafted. In contrast to AM580, neither CD437 nor AC55649 impacted HSC development (Figure 4B and Table S1). These data suggest that the impact of RA signaling on developing HSCs is mediated primarily via RAR-α. To further demonstrate agonist specificity of the effect, E+ cells were treated with ATRA in the absence and presence of the RAR-α-specific antagonist (Ro-415253) (Apfel et al., 1992). As shown in Figure 4C and Table S2, the addition of the antagonist inhibited the effect of ATRA, providing further evidence that the HSC inductive effect of RA signaling is primarily mediated through RAR-α.

As RARs were also expressed in the E10.5 AGM-derived HE progenitors (Figures 1A and 1B), and aldefluor activity was observed at this stage (Figure 1C), we next asked whether RA

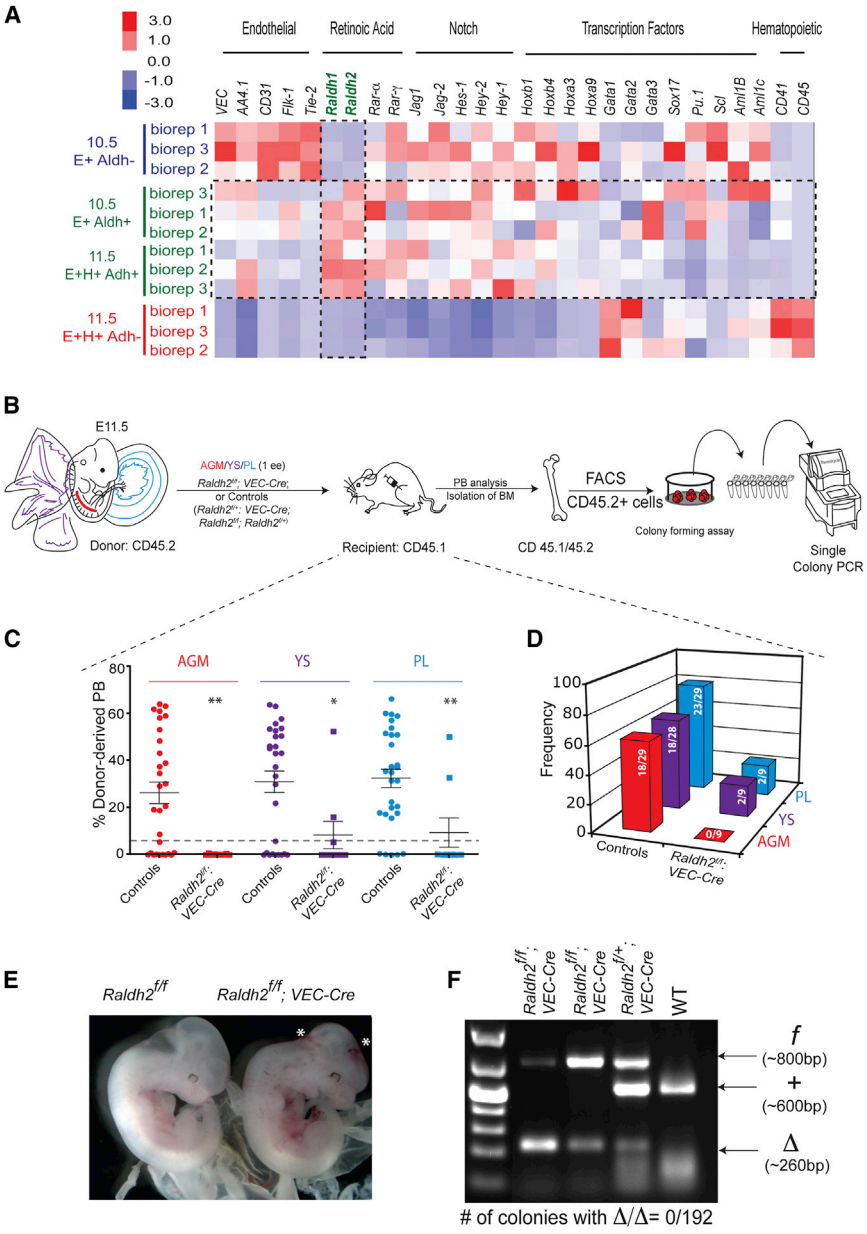

Figure 3. Retinoic Acid Signaling Is Required for the Development of HSCs

(A) Heat-map identifying differential expression of genes based on qRT-PCR analyses from E10.5 E+Aldh− (blue), E+Aldh+ (green), E11.5 E+H+Aldh+ (green) and E+H+Aldh− (red) fractions. Pre-HSC/hemogenic endothelium and HSC containing fractions and *Raldh1* (*Aldh1a1*) and *Raldh2* (*Aldh1a2*) expression are highlighted with dashed lines. Biological replicates are indicated at the left of each row, and genes are indicated on the top of each column.

(B) Schematic for transplantation analyses. Bone marrow-derived CD45.2+ cells, contributed by *Raldh2$^{f/f}$; VEC*-Cre embryos, were analyzed for the presence or absence of a functional *Raldh2* allele.

(C) Analysis of donor-derived PB in mice reconstituted at 1 e.e with HSCs from different regions (AGM, YS, and PL) of *Raldh2$^{f/f}$*; *VEC*-Cre, or control (*Raldh2$^{f/+}$; VEC*-Cre, *Raldh2$^{f/f}$*, or *Raldh2$^{f/+}$*) embryos (*p < 0.05, **p < 0.01, t test). Error bars represent the SEM.

(D) Frequency of mice engrafted with > 5% donor-derived cells with *Raldh2$^{f/f}$; VEC*-Cre or control embryos (*Raldh2$^{f/+}$; VEC* –Cre, or *Raldh2$^{f/f}$*, or *Raldh2$^{f/+}$*).

(E) Morphology of *Raldh2$^{f/f}$; VEC*-Cre and *Raldh2$^{f/f}$* embryos. White asterisks indicate abnormal morphology in the *Raldh2$^{f/f}$; VEC*-Cre embryo.

(F) Polymerase chain reaction (PCR) genotyping of single colonies picked from methylcellulose cultures generated from the bone marrow (CD45.2) of placental cell recipients (CD45.1). The size of the WT (+), floxed (f) and deleted (Δ) *Raldh2* alleles are depicted with arrows. Number (#) of colonies with Δ/Δ genotype is indicated at the bottom. See also Figure S2 and Table S1.

signaling could impact HSC development from HE using the approach outlined in Figure 5A, i. Treatment of the E10.5 E+ population with AM580 resulted in a striking enhancement of HSC potential as shown by both higher levels of donor-derived cells (m = 35.6%, p < 0.05) and increased proportion of repopulated recipients (f = 83.3%) compared to the DMSO control (m = 3.2%, f = 33.3%) (Figure 5B). Culture with CD437 again had no effect (Figure 5B) and AC55649 was not tested given the low level of RAR-β expression at this stage (Figure 1B). Consistent with the transplantation analyses, AA4.1+/VEC+ progenitors cultured with ATRA and AM580 generated more AA4.1+/VEC+ CD45+ cells than those cultured with DMSO-treated cells (Figures S2B and S2C). LDA revealed that treatment with AM580 resulted in an approximate 5-fold increase in HSC numbers compared to the DMSO control (p = 0.000068) (Figure 5C and Tables S1 and S4). The findings from this set of experiments strongly suggest that RA signaling does function to promote HSC development from HE in the AGM region.

Given that the AM580 treatment showed striking HSC inductive effects and that the *Raldh2* deletion resulted in the complete abrogation of HSC development, we next determined if exogenous addition of the RAR-α agonist could rescue the HSC phenotype of *Raldh2$^{f/f}$;VEC*-Cre embryos. To address this, the AGM regions of E10–E10.5 *Raldh2$^{f/f}$;VEC*-Cre embryos were isolated, pooled, and cultured with AM580 or DMSO prior to transplantation (Figure S2D). Treatment with AM580 did appear to rescue some HSC potential as two out of six recipients showed repopulation at 12 weeks posttransplantation. In contrast, none of the recipients that received cells cultured in DMSO were engrafted (Figure S2E). Using the same approach outlined in Figure 3B, we verified the genomic deletion of the *Raldh2* alleles in hematopoietic colonies generated from CD45.2 (donor) cells isolated from the engrafted recipients (data not shown). These findings indicate that AM580 can rescue

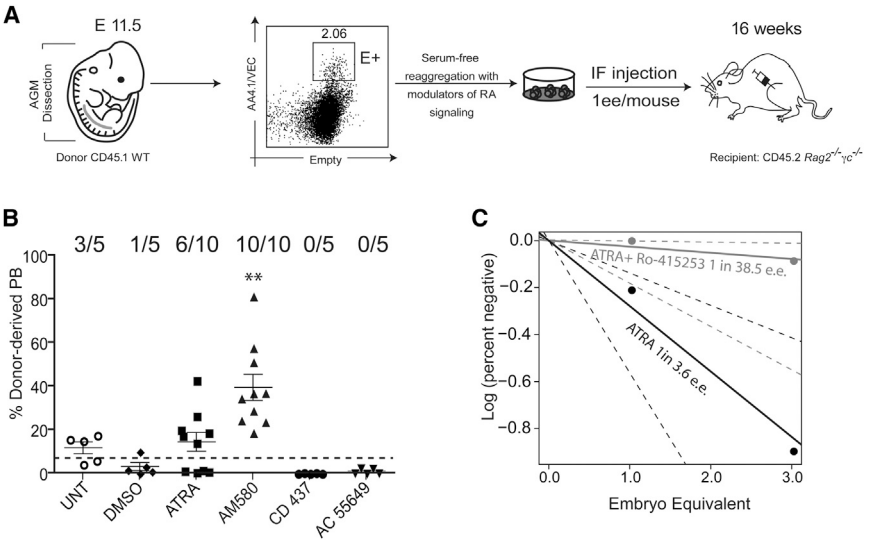

Figure 4. Retinoic Acid Signaling via RAR-α Promotes HSC Development

(A) Transplantation assay scheme. E+ (AA4.1+/VEC+) cells isolated from E11.5 WT embryos (CD45.1) were cultured as aggregates with modulators of RA signaling for ~8 hr, and then transplanted at 1 e.e. intrafemorally into CD45.2 *Rag2*$^{-/-}\gamma c^{-/-}$ recipients.

(B) Engraftment as assessed by flow cytometry on peripheral blood (PB) to detect donor-derived (CD45.1+) cells at 16 weeks or more post-transplantation. Each point represents a single recipient. Repopulated mice are those containing 5% or more donor-derived cells (dotted line). Error bars represent the SEM. Frequency of mice with > 5% donor-derived chimerism in each group is shown at the top of each treatment panel. Significant difference is indicated with asterisks (**p < 0.01, ANOVA, and Dunnett's postmultiple comparison with UNT or DMSO as comparative column).

(C) Limiting dilution analysis (LDA) showing re-populating potential of 1 and 3 e.e. E+ cells treated with ATRA, either in the presence (solid gray circle) or absence (solid black circle) of the RAR-α antagonist Ro-415253 (10 μM). Solid black and gray lines represent the mean of the ATRA, and ATRA+Ro-415253 treatments, respectively, whereas the dotted lines represent 95% confidence interval of the same (p = 0.00389, ELDA). See also Figures S1 and S3 and Tables S1 and S2.

the HSC potential in the Raldh2 null embryos, further demonstrating that the RA pathway is required for HSC development.

As a subpopulation of the E10.5 E+ fraction is aldefluor positive, it is possible that this activity also marks pre-HSCs/HE. To investigate this possibility, the E+Aldh+ and E+Aldh− populations were isolated and cultured with AM580 or DMSO, and subsequently transplanted into recipient mice (Figure 5A,ii). As shown in Figure 5D, the effects of the RAR-α agonist on HSC development were observed predominantly in the E10.5 AGM-derived E+Aldh+ fraction (m = 25%, p < 0.01; f = 77.7%) demonstrating that Aldh activity at this stage does identify the subpopulation of HE that responds to RA signaling and generates HSCs.

RA Signaling Promotes HSC Development through the Transient Downregulation of Wnt Signaling

To better understand the signaling pathways that could regulate HSC development, we next performed microarray profiling, comparing the E11.5 AGM-derived E+H+ (HSC containing) and E+H− (HE) populations. For the initial analyses, we focused on components of key signaling pathways implicated in HSC development and found that the expression levels of β-catenin and many Wnt target genes were significantly lower in E+H+ cells compared to E+H− cells (Figure 6A). Analyses of E10.5 E+ cells treated with either ATRA or AM580 showed that RA signaling led to a downregulation of expression of components of the Wnt signaling pathway in E10.5 E+ cells to levels similar to those found in the E11.5 E+H+ cells (Figure 6B).

To further investigate the status of Wnt signaling in E11.5 AGM, E+H−, E+H+ and E-H+ populations were analyzed by intracellular flow cytometry for their content of total and phosphorylated (Serine 33, 37, and 45; Tyrosine 41) β-catenin, a downstream mediator of the canonical Wnt signaling (Clevers, 2006). The total β-catenin levels were highest in the E+H− population, and gradually decreased with the acquisition of the hematopoietic marker CD45, and the loss of endothelial markers AA4.1 and VEC. Increase in the ratio of phosphorylated β-catenin to total β-catenin, a hallmark for degradation of β-catenin, was predominantly observed in the E+H+ fraction that exclusively contains the developing HSCs (Figures 6C and 6D).

Immunohistochemistry analyses of E11.5 embryos demonstrated the presence of the β-catenin protein in the cells lining the dorsal aorta as well as in the mesenchyme surrounding it (Figure 6E, i). Although the majority of the protein was present at the cell surface and within the cytoplasm, low amounts could be detected in the nucleus, suggesting that the Wnt pathway is active within these cells at this stage of development (Figure 6E, i and ii). In contrast to cells lining the aorta, the hematopoietic cells within it, as well as those that appear to be budding do not contain any detectable β-catenin protein. These observations are consistent with the intracellular flow analyses and support the interpretation that the downregulation of the β-catenin protein correlates with the emergence of HSCs within the dorsal aorta. Collectively, these findings strongly suggest that inhibition of the Wnt pathway is associated with HSC development and that the effects of RA signaling may be mediated through this pathway.

To determine whether the downregulation of Wnt signaling is required for the RA-mediated enhancement of HSCs we treated the E10.5 AGM-derived E+ cells with AM580, the Wnt agonist CHIR99021 (Sato et al., 2004), the Wnt antagonists (iWP2+iCRT14) (Chen et al., 2009a; Gonsalves et al., 2011), or combinations of these modulators prior to transplantation. As expected, treatment with AM580 increased HSC potential (m = 62.5%, p < 0.05, f = 80%) compared to the DMSO control (m = 9.2, f = 40%) (Figures 7A and 7B). If Wnt signaling is downstream

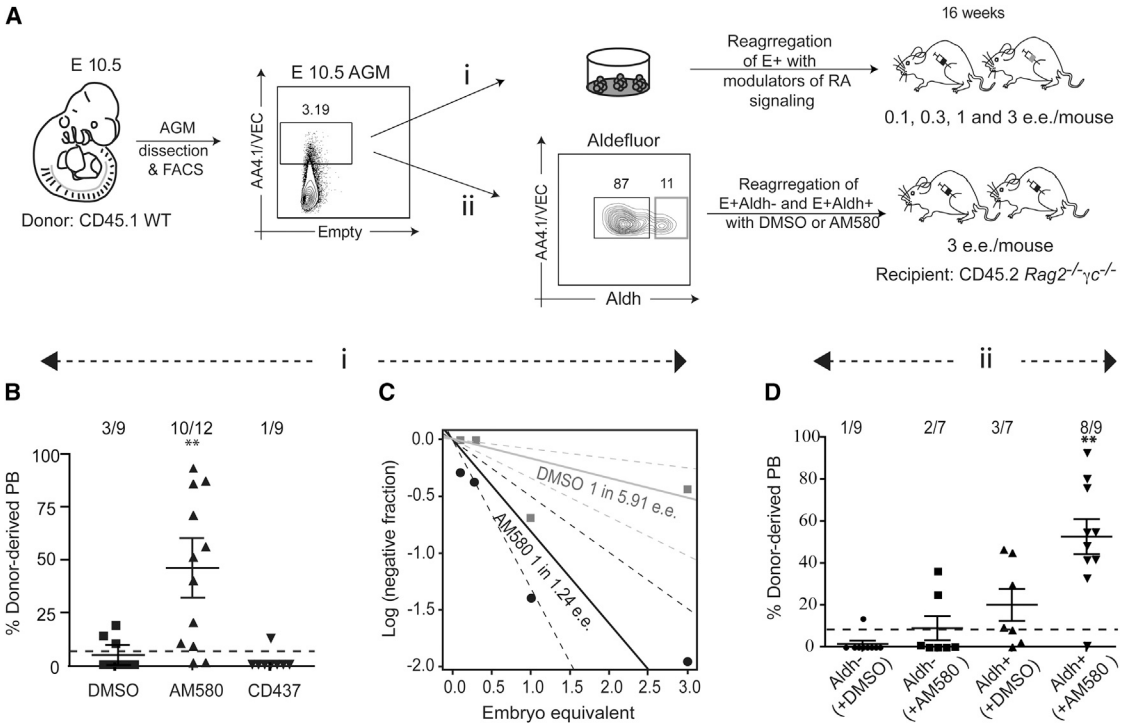

Figure 5. RAR-α Selectively Promotes HSC Development from pre-HSC Progenitors Identified by Aldehyde Dehydrogenase Activity

(A) Schematic of transplantation assays with the different E10.5 AGM-derived isolated cell populations (i and ii) cultured in the presence of RA modulators.

(B) Analysis of donor-derived cells in PB at 16 weeks posttransplantation from 3 e.e. The dashed line represents 5% donor-derived chimerism. Significant difference in percent donor-derived PB cells was observed between AM580 DMSO treated cells (**$p < 0.01$, ANOVA, and Dunnett's postmultiple comparison).

(C) LDA with 0.1, 0.3, 1, and 3 e.e. treated with AM580 (black solid circles, and line) or DMSO (gray solid rectangles, and line). The dashed line represents 95% CI; for both treatments ($p = 6.8 \times 10^{-5}$).

(D) Following the experimental scheme in Figure 5A, ii, analysis of donor-derived PB in mice transplanted with 3 e.e. E10.5 E+Aldh− population treated with DMSO (solid circle) or AM580 (solid rectangle), or E10.5 E+Aldh+ population treated with DMSO (solid triangle) or AM580 (solid inverted triangle). Significant differences from combined DMSO and AM580 treatment of E+Aldh+ and E+Aldh− are indicated with asterisks (**$p < 0.01$, ANOVA, and Dunnett's postmultiple comparison test with Aldh+(+DMSO) as the comparative column). See also Tables S1 and S4.

and downregulated in the presence of RA signaling, we reasoned that activating the pathway should abrogate the HSC inductive effect of RA signaling. Indeed, the effect of RA signaling on HSC development, as measured by frequency and percent engraftment, was dramatically inhibited by the addition of CHIR99021 ($m = 6.5\%$, $p < 0.05$, $f = 40\%$). Alternatively, if Wnt signaling is the primary mediator of the RA signaling pathway, then blocking Wnt signaling alone should mimic HSC inductive effect of RA signaling. Culture with the Wnt inhibitors iWP2+iCRT14 did enhance HSC development in the absence of AM580 ($m = 42.6\%$, $p < 0.05$, $f = 80\%$). The addition of the Wnt inhibitors with AM580 did not show a synergistic effect ($m = 37.5\%$, $p > 0.05$, $f = 80\%$). Collectively, these observations support the interpretation that inhibition of the β-catenin/Wnt pathway, likely via RA signaling, is required for the generation/development of HSCs.

To investigate the interaction between RA and Wnt signaling in more detail, we used a mouse ESC line engineered to contain TOP flash or FOP flash reporters (Lindsley et al., 2006). The mESCs were differentiated using a serum-free protocol to generate a AA4.1+/VEC+ (E+) population that displays many characteristics of the E 10.5 AGM population (Irion et al., 2010; B.C., C.M. Sturgeon, and G.K., unpublished data) (Figure 7C). The mESC-derived E+ cells were isolated at day 6 of differentiation, treated for 24 hr with modulators of the Wnt and RA pathway, and then analyzed for Wnt/β-catenin activity. Addition of ATRA or AM580 in both the presence ($p < 0.01$) or absence ($p < 0.01$) of the Wnt agonist (CHIR99021) significantly reduced the TOP/FOP flash activity, in some cases to levels lower than those detected following the addition of the Wnt antagonists (IWP2 and iCRT14) (Figure 7D). These findings demonstrate that RA signaling can inhibit the Wnt/β-catenin pathway in mESC-derived HE-like progenitors in vitro. To investigate whether the Wnt and RA signaling pathway can directly interact in the ESC-derived progenitors, we stimulated ESC-derived day 6.0 E+ cells for 24 hr with ATRA and performed immunoprecipitation of the β-catenin protein followed by western blot analyses to detect possible association between the RAR-α and β-catenin protein. As shown in Figures 7E and 7F, the RAR-α protein was detected

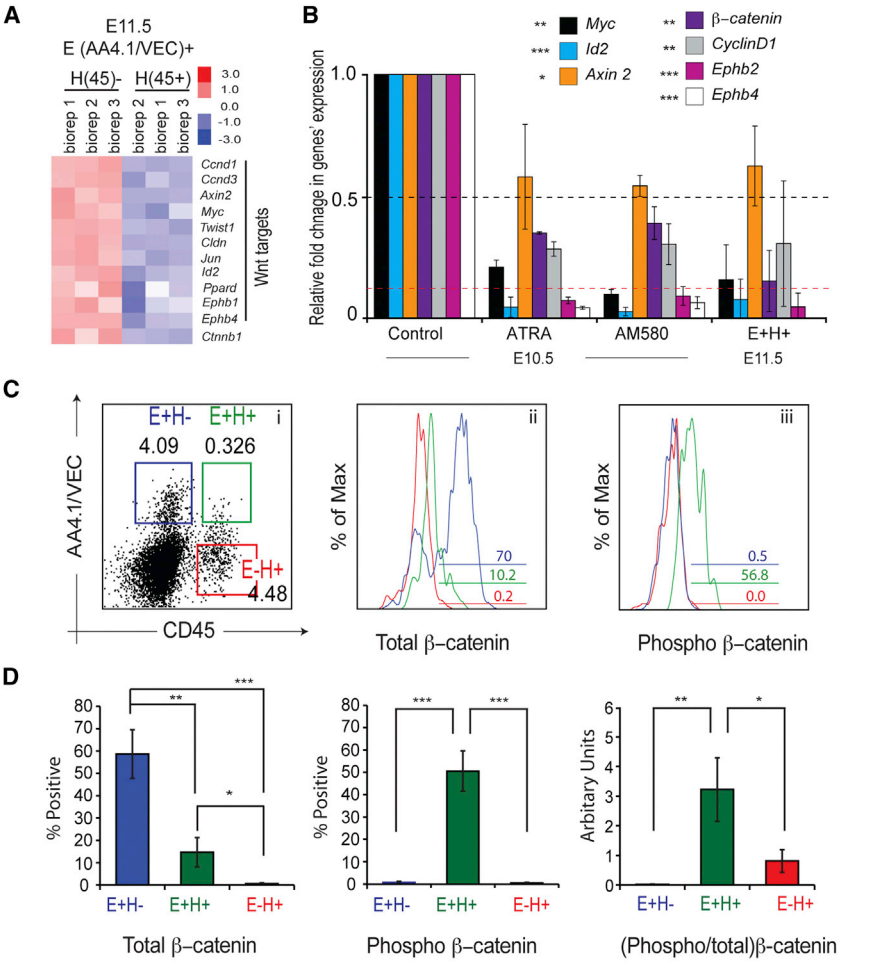

Figure 6. Wnt Signaling Is Downregulated during HSC Development

(A) Heat maps showing differential expression of Wnt target genes between E11.5 E+H− and E+H+ cells. Red and blue indicates upregulation and downregulation, respectively, of a given gene normalized to background intensity. Biological replicates are indicated at the top of each column, and genes are indicated on the right side.

(B) qRT-PCR-based analysis showing the expression of Wnt target genes in pre-HSC (E10.5) and HSC (E11.5) containing fractions with or without exposure to RA signaling modulators. Asterisks indicate significant differences compared to the DMSO control, and error bars represent the SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, t test).

(C) Intracellular flow cytometric analysis of E11.5 AGM-derived (i) E+H− (blue rectangle), E+H+ (green rectangle) and E−H$^+$ (red rectangle) populations to monitor (ii) total β-catenin and (iii) phospho β-catenin, respectively.

(D) Quantitative analysis from three independent experiments to determine the percentage of E+H−, E+H+ and E−H$^+$ cells with total and phospho-β-catenin. Asterisks indicate significant differences, and error bars represent the SEM (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, t test).

(E) Confocal microscopy showing distribution of the β-catenin protein in E11.5 AGM region. (i) The area of interest is highlighted with a white rectangle. Red dashed line indicate nuclear region. (ii) Asterisks indicate cells of interest, whereas area of interest is highlighted with a black rectangle. The lining of the aorta is indicated with dashed white lines. Blue, DAPI; yellow, β-catenin. Bars represent the scale of the respective image.

in the β-catenin-derived precipitate from cells treated with ATRA, indicating that the two proteins can interact in a ligand dependent manner.

DISCUSSION

Understanding the signaling pathways that regulate the key steps in the development of HSCs in the early embryo, including the formation of HE and its transition to a hematopoietic/HSC fate is essential for enabling us to accurately model hematopoietic development in pluripotent stem cell differentiation culture and to ultimately generate functional HSCs in vitro. In this study, we defined a role for the RA signaling pathway in this developmental progression and demonstrate it is essential for HSC development. A key aspect of our studies was the observation that activation of the RA pathway enhances the HSC potential of the E10.5 AA4.1+/VEC+ HE/pre-HSCs and that this effect is restricted to the fraction of the population that displays active aldehyde dehydrogenase activity. These findings identify a signaling pathway that acts directly within

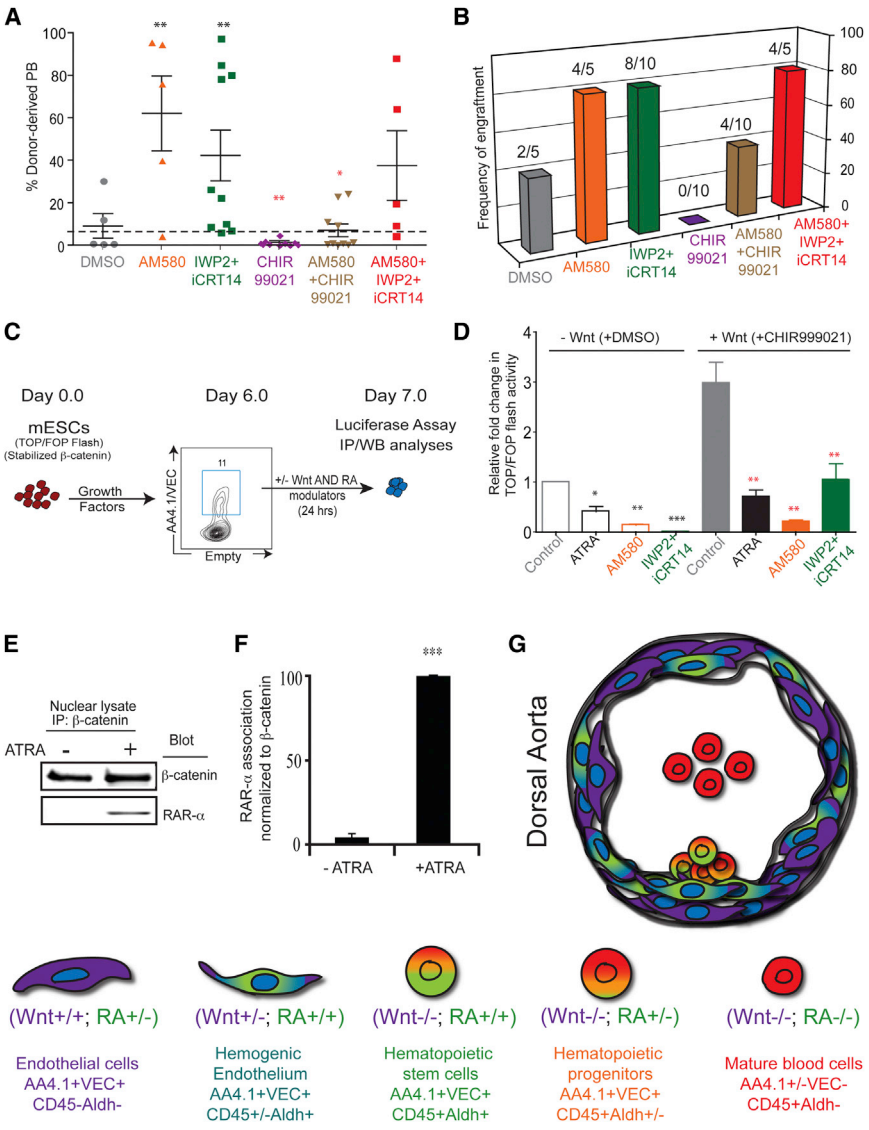

Figure 7. HSC Inductive Effect of Retinoic Acid Signaling Is Modulated by β-catenin Dependent Wnt Signaling

(A) Analysis of donor-derived PB at 16 weeks posttransplantation of the E10.5 E+ population (3 e.e.) treated with modulators of the RA or Wnt signaling pathways. The dashed line represents 5% donor-derived chimerism. Significant differences between cells treated with DMSO and signaling pathway agonists or antagonists (AM580, IWP2+iCRT14, CHIR99021, AM580+CHIR99021, and AM580+ IWP2+iCRT14) are indicated with black asterisks, whereas differences between the AM580-treated cells and cells treated with other pathway modulators are indicated with red asterisks (**p < 0.01, and *p < 0.05 ANOVA, and Dunnett's postmultiple comparison test with DMSO or AM580 as comparative columns).

(B) Frequency of mice displaying > 5% donor-derived PB in response to Wnt and RA modulators.

(C) Schematic of approach used to monitor Wnt signaling in response to treatment with RA signaling modulators in ESC-derived AA4.1+/VEC+ endothelial/hematopoietic progenitors.

(D) Relative fold change in the ratio of TOP/FOP flash activity in response to modulators of RA and Wnt signaling. Significant differences between cells treated with DMSO and signaling pathway agonists or antagonists (ATRA, AM580 and IWP2+iCRT14) in the absence (open columns) or presence (solid columns) of the Wnt agonist CHIR99021 are indicated with black and red asterisks, respectively (***p < 0.001, **p < 0.01, and *p < 0.05; t test).

(E) Blot showing interaction of β-catenin with RAR-α. Samples from nuclear lysates obtained from mESC-derived E+ cells treated with or without RA were immunoprecipitated with anti-β-catenin, and then blotted with anti-RAR-α and anti-β-catenin.

(F) Quantification of the RAR-α and β-catenin protein interaction in the presence or absence of ATRA (***p < 0.001, t test).

(G) Model summarizing interaction of RA and Wnt signaling during commitment of hemogenic endothelium to HSCs and hematopoietic progenitors. See also Table S1.

the isolated HE target population to promote HSC development. Additionally, we provide in vivo and in vitro evidence that the Wnt pathway is a downstream target of RA signaling in this population and show that transient downregulation of β-catenin dependent Wnt signaling is essential for mediating RA's effect on HSC development. Together these observations support a model (Figure 7G) of dynamic regulation of Wnt/β-catenin by RA in HE (AA4.1+/VEC+CD45-Aldh+) progenitors, initiating a sequence of events that promotes rapid maturation/development of the HSC (AA4.1+/VEC+CD45+Aldh+).

Characterization of the signaling pathways that regulate HSC development within the AGM is challenging as this region of the embryo consists of multiple different cell types, with HE and the developing HSCs representing a minority of the entire population. Given this complexity, it is virtually impossible to discriminate between direct and indirect effects of cytokines when added to mixed populations or intact tissues in vitro. To overcome this problem, we focused all of our studies on isolated enriched populations and were able to demonstrate that activation of RA signaling in E10.5 E+ progenitors enhanced their HSC potential by more than 5-fold. Importantly, we further demonstrate that this pro-HSC effect is restricted to the fraction of the population that displays aldehyde dehydrogenase activity. At E11.5, aldehyde dehydrogenase activity defines the HSC subpopulation of VEC/AA4.1+CD45+ cells and distinguished it from the Aldh− fraction that contains the majority of multipotent and GM progenitors. Together, these findings support the interpretation that aldehyde dehydrogenase activity uniquely identifies the HE that will give rise to HSCs and distinguishes it from HE that generates hematopoietic progenitors. The concept of distinct HE giving rise to HSCs and hematopoietic progenitors within the AGM is supported by the findings of Chen and Speck who

showed that the transcriptional regulation of these populations differs (Chen et al., 2011). Both human cord blood and adult mouse marrow-derived HSCs are also Aldh+, suggesting that the capacity to synthesize RA is a feature of HSCs that is maintained beyond the early specification stage (Armstrong et al., 2004; Hess et al., 2004).

Previous studies have provided evidence that the RA pathway does play a role in embryonic hematopoiesis. In an in-vitro-based study using the hESC differentiation model, Yu et al. showed that RA signaling promoted the generation of CD45+ cells, and myeloid and erythroid progenitors from hematovascular progenitors isolated from the differentiation cultures (Yu et al., 2010). In an earlier study, Goldie et al. analyzed the yolk sacs of E9.5 *Raldh2* null embryos and reported defects in a population defined as HE that displayed myeloid and erythroid hematopoietic potential, suggesting that RA signaling played a role in the generation of these progenitors (Goldie et al., 2008). Although these latter observations support a role for RA signaling in embryonic hematopoietic specification, the interpretation of the phenotype is complicated by the fact that *Raldh2* null embryos show broad vascular defects and are embryonic lethal at the stage they were characterized or shortly thereafter. To circumvent this problem, we selectively deleted the *Raldh2* gene in the VEC+ population and demonstrated that *Raldh2* is essential for HSC development. The *Raldh2*$^{f/f}$*;VEC*-Cre deleted embryos did not show dramatic abnormalities and had intact dorsal aorta lined with VEC+ cells, indicating that the absence of HSCs is not due to extensive morphological or endothelial-specific defects within the AGM. Rather, our ex vivo studies point to a role for RA signaling within the Aldh+ HE/pre-HSC population either through promoting proliferation of low numbers of HSC already present or by initiating a sequence of maturation events. The fact that the effect was most pronounced on the E10.5 population, which contains fewer HSCs than the E11.5 fraction, suggests that RA signaling induces maturation of a HE to the HSC.

Through the use of different pathway agonists we were able to demonstrate a striking degree of receptor specificity for the enhancement of HSCs ex vivo and identified RAR-α as the primary receptor mediating this effect. The lack of an effect of the RAR-γ agonist is somewhat unexpected, given that the receptor is expressed in the target population at the appropriate developmental stage. This expression pattern suggests that under certain circumstances, such as in RAR-α null embryos, it may provide a compensatory function to promote HSC development. This interpretation would be consistent with the observation that RAR-α null mice are viable with no gross developmental hematopoietic defects, whereas the RAR-α/RAR-γ compound mutants are embryonic lethal (Mark et al., 2009; Rhinn and Dollé, 2012). Although RAR-α agonist showed a striking HSC inductive effect, neither RAR-α nor RAR-γ expression level varied between the E+H− and E+H+ populations (data not shown). A differential function of these receptors has also been demonstrated in adult hematopoiesis, where RAR-γ, but not RAR-α, has been shown to play a role in the maintenance of the adult HSC population (Purton et al., 2006; Walkley et al., 2007). As observed with the AGM HE, both receptors are expressed in the adult HSCs. We found that all RARs are expressed in the E13.5 AA4.1+ fetal liver population and that both RAR-α and RAR-γ agonists modestly enhanced the repopulation potential of these cells (Figure S3). Collectively, these observations highlight important differences in embryonic, fetal and adult hematopoiesis, and support a model in which different RARs function to regulate HSC development, expansion and maintenance at different stages of development and in adult life.

Our observation that RA signaling downregulates the Wnt pathway is consistent with other studies that have shown similar inhibitory effects on this pathway by retinoid signaling (Easwaran et al., 1999; Xiao et al., 2003). Easwaran et al. (1999) showed that the effect could be mediated via direct interaction of β-catenin with RARs in a retinoid-dependent manner, and/or through RAR competition with TCF for β-catenin binding. We observed a similar interaction between the β-catenin and RAR-α protein in ESC-derived progenitors. A role for Wnt/β-catenin signaling in the generation of HSC within the AGM has been described in both the zebrafish and mouse (Goessling et al., 2009; Ruiz-Herguido et al., 2012). However, in contrast to the findings presented here, these studies provided evidence that Wnt signaling promotes HSC development. Although these observations may at first appear to contradict ours, the differences may be related to temporal changes in the function of the pathway as the studies on the mouse AGM indicate that the requirement for Wnt signaling is stage specific and most pronounced at E10.5 of gestation prior to the peak of HSC development at E11.5 (Ruiz-Herguido et al., 2012). This interpretation is consistent with our findings that the E+H− HE population, at E11.5, displays higher Wnt signaling than the E+H+ HSC population and that β-catenin is present in the cells lining the dorsal aorta but not in the budding cells or the hematopoietic cells within the aorta. Collectively, these observations raise the interesting possibility that signaling through the Wnt pathway is required for the generation of HE, but thereafter needs to be downregulated to promote HSC development (Figure 7G).

In summary, the findings in this study have identified a pivotal role for RA signaling in the generation of HSCs in the early embryo and provide evidence that it mediates this function through the inhibition of the Wnt/β-catenin pathway. Additionally, they show that aldehyde dehydrogenase activity uniquely marks the pre-HSCs and the HSCs within the HE and emerging hematopoietic population, respectively, suggesting that these progenitors display the capacity to synthesize and respond to RA. Together, these observations provide important insights into the stage-specific regulation of embryonic HSC development in vivo and in doing so inform us on key pathways that will need to be manipulated in embryonic stem cell differentiation cultures to bring us one step closer to generating HSCs in vitro.

EXPERIMENTAL PROCEDURES

Further details of experimental procedure can be found in the Extended Experimental Procedures.

Embryo Generation and Transplantation Assay

All experiments with animals were performed according to the University Health Network Animal Resource Center's guidelines. Timed matings were performed to generate embryos, with vaginal plug discovery as embryonic day (E) 0.5. Embryos with 32–40 somite pairs were staged as E10.5, and

42–48 somite pairs as E11.5 for transplantation experiments. Embryos were dissected at either E10.5 or E11.5 stages for isolation of the AGM, and when required, the YS and PL. Desired cell populations or whole organs from embryos were transplanted intrafemorally into recipient mice. Donor-derived PB was analyzed at 16 or 32 weeks posttransplantation assay.

Conditional Deletion of *Raldh2*$^{f/f}$ by *VEC*-Cre

Raldh2$^{f/f}$ and *VEC*-Cre mice were obtained from Dr. Shanthini Socknathan at John Hopkins University, and Dr. Nancy Speck at the University of Pennsylvania, respectively. *Raldh2*$^{f/+}$; *VEC*-Cre mice were generated by crossing of female *Raldh2*$^{f/f}$ with *VEC*-Cre male mice, whereas *Raldh2*$^{f/f}$;*VEC*-Cre conceptus was generated by mating *Raldh2*$^{f/+}$; *VEC*-Cre males with *Raldh2*$^{f/f}$ females.

Aldefluor Staining

Aldefluor staining was performed as described elsewhere (Jones et al., 1995) (Stem Cell Technologies). Briefly, AGM regions were dissected and stained with aldefluor or DEAB+ aldefluor as negative control, at 37°C for 30 min followed by staining with anti-mouse VEC, AA4.1, and CD45 antibodies.

Flow Cytometry

Desired cell populations were washed and Fc receptors were blocked by addition of anti-CD16/32 (eBioscience E03558-1632). Cells were then stained with desired antibodies; anti-VEC (eBioscience E08593-1630), anti-AA4.1 (eBioscience E01895-186), and anti-CD45 (eBioscience E10032-1631). Specific cell-populations were sorted on a FACS Aria (BD Biosciences), whereas they were analyzed on an LSR II flow cytometer (BD Biosciences), and the data were processed with FlowJo 9.5.3 (Tree Star).

Microarray Analyses

Mouse WG-6 v2 Bead chip from Illumina was used to monitor global gene expression from high quality RNA (RNA integrity number > 9.0) extracted from E11.5 E+H+ and E+H− cells. Nonnormalized summary probe profiles (including regular probe profile and control probe profile), with associated probe annotation, were output from Bead Studio. We then performed normexp-by-control background correction, quantile normalization and log2 transformation to the raw data using the neqc function in linear models for microarray data (LIMMA) R package. False discovery rate (FDR) was evaluated using the Benjamini and Hochberg multiple testing procedure. Heat maps were generated using Dchip software with centroid linkage, expression by peaking time and 1- Pearson correlation as significance.

ACCESSION NUMBERS

The GEO accession number for the microarray data is GSE45020.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.08.055.

AUTHOR CONTRIBUTIONS

B.C designed, performed, and analyzed data from all experiments. B.C and A.D performed experiment in Figure 6A. N.I critically reviewed the manuscript. B.C and G.K wrote the manuscript. G.K. supervised the entire study.

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