Lysophosphatidic acid acts as a nutrient-derived developmental cue to regulate early hematopoiesis

Haisen Li¹,†, Rui Yue¹,²,†, Bin Wei³, Ge Gao³, Jiulin Du⁴ & Gang Pei¹,⁵,*

Abstract

Primitive hematopoiesis occurs in the yolk sac blood islands during vertebrate embryogenesis, where abundant phosphatidylcholines (PC) are available as important nutrients for the developing embryo. However, whether these phospholipids also generate developmental cues to promote hematopoiesis is largely unknown. Here, we show that lysophosphatidic acid (LPA), a signaling molecule derived from PC, regulated hemangioblast formation and primitive hematopoiesis. Pharmacological and genetic blockage of LPA receptor 1 (LPAR1) or autotoksin (ATX), a secretory lysophospholipase that catalyzes LPA production, inhibited hematopoietic differentiation of mouse embryonic stem cells and impaired the formation of hemangioblasts. Mechanistic experiments revealed that the regulatory effect of ATX-LPA signaling was mediated by PI3K/Akt-Smad pathway. Furthermore, during in vivo embryogenesis in zebrafish, LPA functioned as a developmental cue for hemangioblast formation and primitive hematopoiesis. Taken together, we identified LPA as an important nutrient-derived developmental cue for primitive hematopoiesis as well as a novel mechanism of hemangioblast regulation.

Keywords embryonic stem cell; hemangioblast; hematopoiesis; LPA; zebrafish

Subject Categories Development & Differentiation; Signal Transduction

DOI 10.15252/embj.201387594 | Received 4 December 2013 | Revised 24 March 2014 | Accepted 10 April 2014 | Published online 14 May 2014

The EMBO Journal (2014) 33: 1383–1396

Introduction

The first wave of hematopoiesis, or primitive hematopoiesis, occurs in the yolk sac blood islands, which is initiated by mesodermal progenitor cells called hemangioblasts that can give rise to both primitive erythrocytes and endothelial cells through asymmetric cell division (Fehling et al., 2003; Orkin & Zon, 2008). The hemangioblasts were initially characterized in vitro using the hematopoietic differentiation model of mouse embryonic stem cells (mESCs) (Kennedy et al., 1997; Choi et al., 1998) and have been more recently identified in the primitive streak of the mouse embryo (Huber et al., 2004). The hemangioblasts exist at a very low frequency and are only detectable in a very short period of time during embryogenesis with the well-known cell-surface marker fetal liver kinase 1 (Flk1) (Ema et al., 2003; Huber et al., 2004). Previously, transcription factors such as Smad1, Scl, Lmo2, Flk1, and Gata2 were shown to regulate the formation of hemangioblasts (Warren et al., 1994; Gering et al., 2003; Lugus et al., 2007; Qian et al., 2007; Zafonte et al., 2007; Liu et al., 2008). Bone morphogenetic protein 4 (BMP4), fibroblast growth factor (FGF), hedgehog, and vascular endothelial growth factor (VEGF) were also demonstrated to regulate the induction of hemangioblasts (Damert et al., 2002; Park et al., 2004; Hochman et al., 2006; Walmsley et al., 2008); however, whether lipid molecules or G protein-coupled receptor (GPCR) signaling can play equally important roles in hemangioblast regulation is still elusive.

The yolk sac contains a large amount of phospholipids such as phosphatidylcholines (PC) to provide nutrition for the developing embryo (Noble & Moore, 1967; Fisher et al., 2002; Freyer & Renfree, 2009). PC are hydrolyzed into lysophosphatidylcholine (LPC) by secreted phospholipase A2 (sPLA2) (Schmitz & Ruebsaamen, 2009), and LPC is further hydrolyzed into LPA by the secretory lysophospholipase ATX (Tokumura et al., 2002; Umezuzo-Goto et al., 2002). LPA is an important signaling molecule that functions by activating its cognate GPCR (Anliker & Chun, 2004). So far, six subtypes of LPA receptor have been identified (LPAR1-6). Lpar1-deficient mice exhibit neonatal lethality partially due to defective suckling (Contos et al., 2000), while lpar3-deficient mice show abnormal embryo spacing and delayed blastocyst implantation (Freyer & Renfree, 2009). In contrast, both lpar2- and lpar4-deficient mice are grossly normal (Estivill-Torrus et al., 2008; Lee et al., 2008). Interestingly, atx-deficient mice died at embryonic day 9.5 with profound vascular defects in the yolk sac (van Meerendonk et al., 2006; Koike et al., 2009). Moreover, catalytic site-mutated atx knock-in mice are also embryonic lethal due to severe vascular defects (Ferry et al., 2007), suggesting that ATX-LPA signaling plays a fundamental role during vascular development. Recently, LPA was reported to regulate definitive erythropoiesis through activating LPAR3 in a hematopoietic

1 State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell biology, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai, China
2 Howard Hughes Medical Institute, Children's Medical Center Research Institute, Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, TX, USA
3 Center for Bioinformatics, State Key Laboratory of Protein and Plant Gene Research, College of Life Sciences, Peking University, Beijing, China
4 Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China
5 Shanghai Key Laboratory of Signaling and Disease Research, School of Life Science and Technology, Tongji University, Shanghai, China

†These authors contributed equally to this work
*Corresponding author. Tel: +86 21 54921372; Fax: +86 21 54921011; E-mails: gpei@sibs.ac.cn; peigang@tongji.edu.cn
stem cell differentiation model (Chiang et al., 2011); however, whether LPA signaling also regulates primitive hematopoiesis during early development remains unclear.

In this study, we identified LPA as a critical regulator of hemangioblast formation and primitive hematopoiesis by analyzing the hematopoietic differentiation of mESCs and the zebrafish embryogenesis. Furthermore, we showed that LPAR1, but not LPAR3, mediates this effect by activating PI3K/Akt-Smad pathway, highlighting the role of LPA as a nutrient-derived developmental cue during early development.

## Results

### LPA regulates hematopoietic differentiation

Previously, we had successfully identified F2r as a critical regulator of hematopoiesis through a microarray analysis (Hailesellasse Sene et al., 2007; Yue et al., 2012). In addition to F2r, we also found that lpar1 and lpar3 were significantly up-regulated during in vitro hematopoietic differentiation of mESCs (Supplementary Fig S1A and B). Interestingly, atx expression peaked at day 4 of EB formation before lpar1 and lpar3 (Supplementary Fig S1C), which coincided with the reported window of hemangioblast formation and blood fate specification (between day 3 and day 4 of hematopoietic differentiation) (Kennedy et al., 1997), indicating that ATX-LPA signaling may play a role during hematopoietic differentiation.

Next, we used both pharmacological and genetic approaches to modulate LPA signaling during hematopoietic differentiation. Treatment of two structurally different LPAR1/3-specific antagonists, Ki16425 and VPC32183, dose-dependently reduced CD41+ hematopoietic cell percentage in day 6 EBs (Fig 1A–B and Supplementary Fig S2A–B). Consistently, quantitative real-time PCR (qPCR) analyses showed that hematopoietic transcription factors gata1, scl, runx1, cmyb as well as, beta-h1 and beta-major hemoglobins were all down-regulated after LPAR1/3 antagonist treatment (Fig 1C and Supplementary Fig S2C). Methylcellulose colony-forming cell assay (M3434) showed that LPAR1/3 antagonism significantly reduced the primitive erythroid colony numbers (Ery-P) (Fig 1D and Supplementary Fig S2D), as well as the definitive erythroid (cfu-E) and granulocyte/monocyte (cfu-G/M/GM) colony numbers (Fig 1E and Supplementary Fig S2E). To rule out the possibility that the inhibition of hematopoietic differentiation was caused by increased apoptosis, day 6 EBs were dissociated and stained with Annexin-V and PI. Flow cytometry analyses revealed that LPAR1/3 antagonism did not significantly change the percentage of cells that are undergoing apoptosis (Supplementary Fig S3A–D). Finally, we explored the role of ATX during hematopoietic differentiation by using the ATX inhibitor HA130 in a serum-free differentiation medium (Gadue et al., 2006), since serum contains high levels of LPA. HA130 significantly inhibited CD41+ cell percentage in a dose-dependent manner (Fig 1F) and down-regulated the expression of hematopoietic markers (Fig 1G). In addition, HA130 also notably reduced the Ery-p (Fig 1H), cfu-E, and cfu-G/M/GM colony numbers (Fig 1I).

Since Ki16425 and VPC32183 block both LPAR1 and LPAR3, we further attempted to clarify the downstream receptor by using small interference RNA (siRNA)-mediated knockdown. Stable mESC lines harboring lpar1 and/or lpar3 siRNAs were constructed using lentivirus infection followed by flow cytometry sorting. The knockdown efficiency was determined by qPCR (Fig 2A). Genetic inhibition of lpar1 significantly decreased CD41+ cell percentage (Fig 2B), hematopoietic marker expression (Fig 2C), and colony-forming cell numbers (Fig 2D and E). In contrast, inhibition of lpar3 showed no significant changes, and simultaneous knockdown of lpar1 and lpar3 demonstrated no synergistic effects compared to lpar1 knockdown (Fig 2B–E). We also established a mESC line stably expressing the Atx siRNA and differentiated it in a serum-free medium (Fig 2F). Consistently, knockdown of atx also significantly reduced CD41+ cell percentage, hematopoietic marker expression, and the colony-forming cell numbers (Fig 2G–J). These results not only confirmed the pharmacological blockage data, but also indicated that LPAR1 mediates the downstream effects of LPA to regulate hematopoietic differentiation.

To determine whether LPA is sufficient to promote hematopoietic differentiation, we utilized a serum-free system to minimize the effect of serum-derived LPA (Chiang et al., 2011). LPA significantly increased CD41+ cell percentage and hematopoietic marker expression (Supplementary Fig S4A–C), which was abolished by Ki16425 or VPC32183 treatment (Supplementary Fig S4D). Similarly, the effect of LPA was also abolished after lpar1 knockdown (Supplementary Fig S4E), indicating that LPA promotes hematopoietic differentiation via LPAR1. In contrast, treatment of sphingosine-1-phosphate (SIP), another prototypical lysoosphospholipid, or SIP receptor agonist FTY720P, did not affect CD41+ cell percentage (Supplementary Fig S5A and B). Taken together, these data provide evidence that LPA regulates hematopoietic differentiation in vitro.

### LPA regulates hemangioblast formation

During in vitro hematopoietic differentiation, mESCs first generate flk1+ hemangioblasts, which then give rise to CD41+ hematopoietic

---

**Figure 1. Pharmacological blockage of ATX-LPA signaling inhibits hematopoietic differentiation.**

A. Representative flow cytometry data for CD41 staining in day 6 EBs, treated with DMSO or 30 μM Ki16425 from day 2 to day 6.

B. Dose effect of Ki16425 on mESC hematopoietic differentiation. The relative fold changes of CD41+ cell percentage were shown (n = 5).

C. qPCR analyses of hematopoietic marker expressions (n = 4).

D, E. Methylcellulose colony-forming cell assay (M3434). Primitive erythroid colonies were scored 6 days after culture in M3434 (D) (n = 4). Definitive colonies were scored 10 days after culture in M3434 (E) (n = 4). Insets show average distribution of hematopoietic colonies. Ery-P: primitive erythroid; cfu-E: definitive erythroid; cfu-G/M/GM: granulocyes/macrophages/granulocyte macrophages; cfu-GEMM: granulocyte/erythroid/megakaryocyte/dendrocyte.

F. Dose effect of HA130 on CD41+ cell percentage (n = 3).

G. qPCR analyses of hematopoietic marker expressions (n = 4).

H, I. Methylcellulose colony-forming cell assay (M3434). Primitive (H) and definitive (I) colonies were cultured and scored as in (D) and (E) (n = 3 per group).

Data information: Data shown are means ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001 versus the corresponding control.
The EMBO Journal

Vol 33 | No 12 | 2014

Haisen Li et al

LPA signaling in early blood development

Published online: May 14, 2014

© 2014 The Authors

A

Relative mRNA Level

Ctrl RNAi

Lpar1 RNAi

B

Fold % CD41+

Ctrl RNAi

Lpar1 RNAi

C

Relative mRNA Level

Ctrl RNAi

Lpar1 RNAi + Lpar3 RNAi

Lpar3 RNAi

D

Fold change in c.f.u.

Ctrl RNAi

Lpar1 RNAi

Lpar1 RNAi + Lpar3 RNAi

Lpar3 RNAi

E

Average c.f.u. per 100,000 cells

Ctrl RNAi

Lpar1 RNAi

Lpar1 RNAi + Lpar3 RNAi

Lpar3 RNAi

F

Relative mRNA Level

Ctrl RNAi

Atx RNAi

G

Fold % CD41+

Ctrl RNAi

Atx RNAi

H

Relative mRNA Level

Ctrl RNAi

Atx RNAi

I

Fold change in Ery-P

Ctrl RNAi

Atx RNAi

J

Fold change in c.f.u.

Ctrl RNAi

Atx RNAi
progenitor cells and more mature hematopoietic cell types (Eilken et al., 2009; Lanrinc et al., 2009). Thus, we went on to determine whether LPA signaling regulates hemangioblast formation. Ki16425 and VPC32183 treatment significantly reduced flk1+ cell percentage in day 4 EBs (Fig 3A–B and Supplementary Fig S2F–G). Consistently, qPCR analyses showed that LPAR1/3 antagonism notably inhibited the expression of flk1 as well as other hematopoietic transcription factors. In contrast, the endoderm marker lamb1, pan-mesoderm marker brachyury, and ectoderm marker beta-tub3 were not affected, suggesting that the specification of three germ layers was not generally affected (Fig 3C and Supplementary Fig S2H). In addition, we performed blast colony-forming cell (BL-CFC) assay to functionally measure hemangioblast numbers and found that LPAR1/3 antagonism led to significantly reduced BL-CFCs (Fig 3D and Supplementary Fig S2I). The inhibitory effect of LPAR1/3 antagonism on hemangioblast formation was not a consequence of increased cell apoptosis (Supplementary Fig S3E–H). Similarly, ATX inhibitor HA130 also significantly impaired hemangioblast formation in day 4 EBs (Fig 3E–G).

Consistent with the pharmacological results, siRNA-mediated knockdown of lpar1 dramatically reduced flk1+ cell percentage, flk1 and hematopoietic marker expression, and the BL-CFC numbers in day 4 EBs, whereas knockdown of lpar3 did not show any inhibitory effects (Fig 4A–C). The fact that LPAR3 had no effects in regulating hematopoiesis in vitro is probably due to the late onset of lpar3 expression during EB differentiation (Supplementary Fig S1B). Similarly, knockdown of atx also significantly compromised hemangioblast formation (Fig 4D–F). Furthermore, high concentrations of LPA (10–30 μM) significantly increased flk1+ cell percentage in the serum-free system (Supplementary Fig S6A), whereas treatment of S1P or FTY720P did not affect flk1+ cell percentage (Supplementary Fig S6B and C). Collectively, these results showed that LPA signaling is both required and sufficient for hemangioblast formation.

ATX-LPA signaling regulates hematopoietic differentiation through PI3K/Akt-Smad pathway

Next, we attempted to illustrate the downstream mechanisms of LPA signaling during hematopoietic differentiation. To test whether there is cross talk between LPA signaling and known hematopoietic pathways, we applied the inhibitors of several hematopoietic pathways in combination with LPA in a serum-free differentiation system and analyzed CD41+ hematopoietic cell percentage. BMP pathway inhibitor dorsomorphin significantly inhibited hematopoietic differentiation, which was consistent with the fact that BMP signal is critical for mesoderm induction (Soderberg et al., 2009). In contrast, inhibition of FGF pathway, Hedgehog pathway, or nitric oxide pathway did not notably abolish the effects of LPA (Supplementary Fig S8A). Dorsomorphin was previously shown to inhibit VEGF receptor 2 (Flk1) signaling (Hao et al., 2010); however, VEGF receptor inhibitor axitinib did not abolish the effect of LPA treatment (Supplementary Fig S8B), suggesting that there is no cross talk between LPA signaling and Flk1 signaling. LPA was also shown to activate β-catenin, a key downstream component of Wnt signaling pathway, during definitive erythropoiesis (Chiang et al., 2011). However, β-catenin inhibitor quercetin also did not abolish the effect of LPA (Supplementary Fig S8C), indicating that LPA signaling and Wnt pathway do not cross talk during hematopoietic differentiation.

After stimulation by LPA, LPAR1 binds to G proteins such as G13, Gq, and G12/13, which further activates PI3K/Akt, PKC, or RhoA/ Rock pathway, respectively (Fukushima et al., 1998, 2000; Contos et al., 2000). Interestingly, Gi inhibitor PTX significantly abolished the effect of LPA, whereas ROCK, Rac1, and PKC inhibitors did not affect LPA signaling (Fig 5A and Supplementary Fig S8D). The effect of LPA was also abolished by PI3K inhibitors LY294002, wortmannin, and Akt inhibitor AKT VIII (Fig 5B). Consistently, Western blot analysis showed that lpar1, but not lpar3, knockdown led to decreased Akt phosphorylation in day 4 EBs, while simultaneous knockdown of both lpar1 and lpar3 had no synergistic effects (Fig 5C).

Previous studies demonstrated that Smad1 stimulates hemangioblast formation as well as hematopoietic development (Zafonte et al., 2007; Cook et al., 2011; Zhang et al., 2011). Since Akt was known to positively regulate Smad1 phosphorylation (Jin et al., 2011), we hypothesized that Smad1 may function as the downstream mediator of ATX-LPA signaling. Indeed, knockdown of lpar1, but not lpar3, significantly decreased Smad1 phosphorylation (Fig 5D). Importantly, LPA significantly increased Akt phosphorylation and Smad1 phosphorylation, which could be abolished by PI3K inhibitor or Akt inhibitor, while Erk1/2 phosphorylation was not affected (Fig 5E). In contrast, LPAR1 antagonist Ki16425 significantly decreased Akt and Smad1 phosphorylation, but not Erk1/2 phosphorylation, while over-expressing the constitutively active form of Akt (CA-AKT) led to opposite effects and partially rescued the inhibitory effects of Ki16425 on Smad1 phosphorylation (Fig 5F). Taken together, these data demonstrated that PI3K/Akt-Smad pathway mediates LPA signaling during hematopoietic differentiation.
Figure 3. Pharmacological blockage of ATX-LPA signaling inhibits hemangioblast formation.

A Representative flow cytometry data for Flk1 staining in day 4 whole EBs. EBs were treated with DMSO or 30 μM Ki16425 from day 2 to day 4 and analyzed by flow cytometry.

B Effect of Ki16425 treatment on Flk1+ cell percentage (n = 5).

C qPCR analyses of hematopoietic and germ layer marker expressions (n = 4). Endoderm marker: lamβ1; mesoderm marker: brachyury; ectoderm marker: beta-tub3.

D BL-CFC assay. Day 4 EBs treated as in (A) were digested with trypsin and cultured in BL-CFC medium. Blast colonies were identified and scored by their distinctive morphology 4 days later (n = 4).

E Effect of HA130 on Flk1+ cell percentage (n = 4).

F qPCR analyses of hematopoietic and germ layer marker expressions (n = 3).

G BL-CFC assay (n = 3). Blast colonies were cultured and scored as in (D).

Data information: Data shown are means ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001 versus the corresponding control.
**Figure 4.** Genetic blockage of ATX-LPA signaling inhibits hemangioblast formation.

A  Effects of Lpar1 and/or Lpar3 knockdown on Flk1+ cell percentage in day 4 whole EBs (n = 4).
B  qPCR analyses of hematopoietic and germ layer marker expressions (n = 4).
C  BL-CFC assay. Day 4 EBs were digested with trypsin and cultured in BL-CFC medium. Blast colonies were identified and scored 4 days later (n = 3).
D  Effect of Atx knockdown on Flk1+ cell percentage (n = 3).
E  qPCR analyses of hematopoietic and germ layer marker expressions (n = 4).
F  BL-CFC assay (n = 4). Blast colonies were cultured and scored as in (C).

Data information: Data shown are means ± s.e.m., *P < 0.05, **P < 0.01, ***P < 0.001 versus the corresponding control.
LPA signaling in early blood development

A

B

C

D

E

F

The EMBO Journal

Fold % CD41+

Relative protein level

Relative protein level

Relative protein level

Relative protein level

Relative protein level
ATX-LPA signaling regulates primitive hematopoiesis during zebrafish embryogenesis

To explore the effect of ATX-LPA signaling on hematopoiesis in vivo, we detected the expression changes of lpar1, lpar3, and atx during zebrafish embryogenesis by qPCR analysis. Lpar1 was markedly up-regulated from 12 hours post-fertilization (hpf) (Supplementary Fig S1D), whereas lpar3 first peaked at 6 hpf and then gradually increased after 24 hpf (Supplementary Fig S1E), which was identical to the previously reported pattern of zebrafish lpar3 (Lai et al, 2012). Atx was dramatically up-regulated after 12 hpf (Supplementary Fig S1F).

Next, we used both pharmacological and genetic approaches to test the role of ATX-LPA signaling during zebrafish hematopoiesis. Whole mount in situ hybridization (WISH) analyses showed that Ki16425 treatment dramatically decreased the expression of hemangioblast markers lmo2 and scl in the lateral-ventral mesendoderm at 12 hpf (Fig 6A, n = 28/29; Fig 6B, n = 17/19; Fig 6C, n = 16/21; Fig 6D, n = 17/26). Consistently, Ki16425 also decreased the expression of primitive hematopoietic markers gata1 and scl in the intermediate cell mass (ICM) at 24 hpf (Fig 6E, n = 16/19; Fig 6F, n = 17/20; Fig 6G, n = 20/30; Fig 6H, n = 19/26), as well as primitive myeloid markers l-plastin and mpo at 26 hpf (Supplementary Fig S11A, n = 13/14; Supplementary Fig S11B, n = 18/19; Supplementary Fig S11C, n = 20/27; Supplementary Fig S11D, n = 19/23). Recently, RAGE (receptor for advanced glycation end products) was shown to be a non-GPCR receptor for LPA (Rai et al, 2012). However, RAGE antagonist FPS-ZM1 did not affect hematopoietic development (Supplementary Fig S10A, n = 21/26; Supplementary Fig S10B, n = 20/28; Supplementary Fig S10C, n = 14/19; Supplementary Fig S10D, n = 15/18; Supplementary Fig S10E, n = 31/34; Supplementary Fig S10F, n = 32/35; Supplementary Fig S10G, n = 28/35; Supplementary Fig S10H, n = 17/22).

In addition, we also injected the morpholino antisense oligonucleotides (MOs) at one-cell stage to block ATX-LPA pathway at the translational level. The specificity of Lpar1 MO was confirmed by a reporter assay (Supplementary Fig S9A–D). 6 ng Lpar1 MO injection remarkably decreased the expression of hemangioblast markers lmo2 and scl at 12 hpf (Fig 6I, n = 48/53; Fig 6J, n = 31/35; Fig 6L, n = 56/67; Fig 6M, n = 39/50), ICM markers gata1 and scl at 24 hpf (Fig 6O, n = 51/56; Fig 6P, n = 31/36; Fig 6R, n = 43/52; Fig 6S, n = 42/46) and primitive myeloid markers l-plastin and mpo at 26 hpf (Supplementary Fig S11E, n = 52/60; Supplementary Fig S11F, n = 53/66; Supplementary Fig S11H, n = 42/50; Supplementary Fig S11I, n = 36/45). Importantly, these defects could be largely restored by co-injection of lpar1 mRNA (Supplementary Fig S12C, n = 47/48; Supplementary Fig S12F, n = 34/37; Supplementary Fig S12G, n = 32/35; Supplementary Fig S12J, n = 30/37; Supplementary Fig S12K, n = 19/23; Supplementary Fig S12L, n = 39/42). In contrast, 2.5 ng lpar3 MO injection (Chiang et al, 2011) had no such inhibitory effects (Fig 6K, n = 30/34; Fig 6N, n = 37/43; Fig 6O, n = 51/58; Fig 6P, n = 45/55; Supplementary Fig S11G, n = 22/31; Supplementary Fig S11H, n = 34/45). Increasing the Lpar3 MO dose up to 5 ng caused developmental delay and reduced the expression of hemangioblast markers lmo2 and scl at 12 hpf (Supplementary Fig S10L, n = 64/65; Supplementary Fig S10M, n = 32/35; Supplementary Fig S10N, n = 37/39; Supplementary Fig S10O, n = 22/28), but did not alter the expression of ICM markers gata1 and scl at 24 hpf (Supplementary Fig S11M, n = 27/27; Supplementary Fig S11N, n = 29/29; Supplementary Fig S11O, n = 36/36; Supplementary Fig S11P, n = 31/32). Importantly, 0.5 ng Atx MO injection also decreased the expression of lmo2 and scl at 12 hpf (Fig 6L, n = 22/38; Fig 6N, n = 26/29; Fig 6P, n = 18/26; Fig 6R, n = 20/23), gata1, and scl at 24 hpf (Fig 6Y, n = 30/44; Fig 6Yi, n = 17/26; Fig 6Zi, n = 23/34; Fig 6Zii, n = 15/19), as well as l-plastin and mpo at 26 hpf (Supplementary Fig S11K, n = 59/66; Supplementary Fig S11L, n = 53/61; Supplementary Fig S11M, n = 15/17; Supplementary Fig S11N, n = 12/14). The specificity of Atx MO was verified (Supplementary Fig S9E–H). Collectively, these results suggested that ATX-LPA signaling is necessary for hemangioblast formation and primitive hematopoiesis in vivo.

Discussion

Hematopoietic development is controlled by the concerted actions of both intrinsic transcription factors, as well as extracellular signals generated by the local environment. In this study, we found that ATX-LPA signaling functions as an evolutionarily conserved pathway that is critically involved in hemangioblast formation and primitive hematopoiesis. Since LPA is a classical metabolite of PC, a major nutrient in the early hematopoietic microenvironment, our results unraveled the morphogenic role of early nutrients and a GPCR-mediated extracellular regulatory mechanism of hemangioblast formation.
Lysophospholipids, including LPA and S1P, have been previously implicated in cardiovascular development (Birgbauer & Chun, 2006). S1P regulates cardiac differentiation of mouse ES cells and vascular maturation in mouse (Liu et al, 2000; Allende et al, 2003; Sachinidis et al, 2003). In addition, SIP and LPA are also shown to be critical for vascular development and stabilization in zebrafish (Yukiura et al, 2011; Gaengel et al, 2012; Lai et al, 2012). Interestingly, 0.5 ng Atx MO injection decreased the expression of endothelial markers flt-1 and flk1 in the intersegmental vessel (ISV) (Supplementary Fig S13A, n = 29/32; Supplementary Fig S13B, n = 22/28; Supplementary Fig S13C, n = 13/13; Supplementary Fig S13D, n = 12/14) at 22 hpf, while higher doses of Atx MO led to embryonic lethality and completely abolished the hemangioblast markers (Unpublished observations), further indicating that ATX regulates hemangioblast specification. The discrepancy between our results and a previous study that Atx morphants had only defects in segmental artery sprouting (Yukiura et al, 2011) is possibly due to the fact that we use different sequences of Atx MO. Furthermore, our observations are consistent with the lethal phenotype of atx-deficient mice that were associated with vascular defects in the yolk sac (van Meeteren et al, 2006; Koike et al, 2009). Since hemangioblast is the common progenitor giving rise to both hematopoietic and endothelial lineages, our results suggested that LPA participates in both vascular and hematopoietic development through hemangioblast regulation. Future studies are needed to carefully examine whether there are hemangioblast defects associated with atx or lpar1 knockout mice during embryonic development.

LPA can be generated both intracellularly by cytosolic phospholipase A2 (cPLA2) and extracellularly by sPLA2 or ATX (Mills & Moolenaar, 2003). In contrast to ATX, cPLA2 and sPLA2 generate LPA by hydrolyzing phosphatidic acid (PA). The intracellular LPA may not play an important role during hematopoietic differentiation, since inhibition of ATX or LPAR1 only affects the extracellular LPA signaling. Consistently, cPLA2 inhibitor OBAA did not affect hematopoietic differentiation (Supplementary Figs S5C and S7A). Similarly, sPLA2 inhibitor BPB also did not affect hematopoietic differentiation (Supplementary Figs S5E and S7C), suggesting that apoptotic cell membrane is not the main source of PC.

The transient nature of hemangioblast in vivo makes it particularly challenging to define its underlying regulatory mechanisms. Thus, the embryonic stem cell differentiation system has been used to study hemangioblast in vitro (Kennedy et al, 1997; Park et al, 2005). The BMP4 signaling is well known to be required for hemangioblast formation (Johansson & Wiles, 1995; Winnier et al, 1995; Park et al, 2004; Nostro et al, 2008). BMP4 stimulation activates the phosphorylation of Smad1/5/8, which then translocates to the nucleus to initiate downstream transcription (Feng & Derynck, 2005; Soderberg et al, 2009). Here, we showed that LPA signaling activates PI3K/Akt pathway to promote the phosphorylation of Smad1/5/8 and that LPA and BMP4 have synergistic effects in promoting hematopoietic differentiation (Supplementary Fig S4F). Consistently, LPAR1 antagonist VPC32183 significantly reduced Smad1 phosphorylation in the presence or absence of BMP4 (Supplementary Fig S8E). In addition, BMP pathway inhibitor dramatically decreased Smad1 phosphorylation without affecting AKT phosphorylation and abolished the effect of LPA (Supplementary Fig S8F). These data highlighted the role of Smad in the cross talk between LPA and BMP4 signaling.

In summary, our study demonstrates that nutrient-derived LPA signaling specifies hematopoietic development through hemangioblast regulation and shed light on the pivotal role of GPCR and lipid metabolism during early development. Interestingly, insufficient or excessive nutrition during pregnancy was shown to increase the risk of metabolic syndromes in the adulthood (Guo & Jen, 1995; Hales & Barker, 2001; Khan et al, 2005). Thus, it is intriguing to examine whether altered metabolism of yolk sac lipids contributes to the later development of adult metabolic or hematopoietic diseases in the future.

Materials and Methods
ES cell culture and differentiation
E14.1 mouse ES cells were cultured and differentiated as previously described (McKinney-Freeman et al, 2008). Briefly, undifferentiated mESCs were maintained in pre-differentiation medium 2 days before hematopoietic differentiation [15% FBS, 1 mM sodium pyruvate (Sigma), 2 mM glutamine (Invitrogen), 0.1 mM non-essential amino acid (Merck Millipore), 10 ng/ml LIF, 100 µM monothioglycerol (Sigma) with 100 U/ml penicillin and 100 µg/ml streptomycin in IMDM (Sigma)]. To form hematopoietic EBs, mESCs were trypsinized into single cells, diluted at 100 mESCs/15 µl differentiation medium [200 µg/ml holo-transferrin (Calbiochem), 4.5 mM monothioglycerol, 15% FBS, 0.5 mM ascorbic acid (Sigma), 2 mM glutamine with 100 U/ml penicillin and 100 µg/ml streptomycin in IMDM], and inverted cultured for 2 days. EBs were then collected and cultured with rotation in 35 mm Petri dishes. Fresh differentiation medium was changed every 2 days. The serum-free differentiation Medium was changed every 2 days. The serum-free differentiation

Figure 6. ATX-LPA signaling is required for hemangioblast formation and primitive hematopoiesis in zebrafish.

A–H WISH analyses for embryos treated with K16425. Zebrafish embryos treated with DMSO or 30 µM K16425 were hybridized with riboprobes to hemangioblast markers lmo2 (A and B) and sci (C and D) at 12 hpf, or with riboprobes to primitive hematopoietic markers gata1 (E and F) and sci (G and H) at 24 hpf. Embryos were dorsal views with anterior to the top (A–D), or lateral views with anterior to the left (E–H).

J–T WISH analyses for embryos injected with lpar1 or lpar3 MO. Zebrafish embryos injected with 4 ng Ctrl MO, 6 ng lpar1 MO, or 2.5 ng lpar3 MO at one-cell stage were hybridized with riboprobes to hemangioblast markers lmo2 (J–K) and sci (L–N) at 12 hpf, or with riboprobes to primitive hematopoietic markers gata1 (O–Q) and sci (R–T) at 24 hpf. Embryos were dorsal views with anterior to the top (J–N) or lateral views with anterior to the left (O–T).

U–Zii WISH analyses for embryos injected with Atx MO. Zebrafish embryos injected with 0.5 µg Ctrl MO or 0.5 ng Atx MO at one-cell stage were hybridized with riboprobes to hemangioblast markers lmo2 (U and V) and sci (W and X) at 12 hpf, or with riboprobes to primitive hematopoietic markers gata2 (Yi and Yii) and sci (Zi and Zii) at 24 hpf. Embryos were dorsal views with anterior to the top (U–X) or lateral views with anterior to the left (Yi–Zii).
medium was also used for LPA- and ATX-related experiments (0.5 × N2/B27 supplements (Invitrogen), 0.05% BSA, 2 mM glutamine, 0.5 mM ascorbic acid, 4.5 mM monothioglycerol, 10 ng/ml BMP4 (R&D Systems), 20 ng/ml Activin A (PeproTech) with 100 U/ml penicillin and 100 µg/ml streptomycin in IMDM/Ham’s F12).

Colony assays

For the hematopoietic progenitor assay, EBs were dissociated by trypsin to single cell suspension and plated in methylcellulose-based medium containing hematopoietic growth factors according to the manufacturer’s instructions (M3434; STEMCELL Technologies). Blast cell colonies (BL-CFC) were assayed as previously described (Kennedy et al., 1997). Colonies were counted under Olympus CK30-F200 microscope (Olympus, Japan) at 10X objective lense.

Real-time quantitative PCR

Total RNAs were extracted from the whole EBs using TRIzol reagent (Sigma). RNA was reverse transcribed using random hexamers and M-MLV Reverse Transcriptase (Promega). 2 × JumpStart Taq ReadyMix (Sigma) and EvaGreen Dye (Biotium) were used for the PCR reactions, with a Stratagene MX3000P (Agilent Technologies) PCR machine. The relative expression values were normalized against the internal control gapdh. Primer sequences are provided in Supplementary Table S1.

Flow cytometry

EB cells were resuspended in PBS containing 1% (wt/vol) BSA. Cells were stained with PE-conjugated anti-FLK1 (555308, BD Biosciences) or APC-conjugated anti-CD41 (17-0411-82, eBioscience) or APC-conjugated anti-CD41 (17-0411-82, eBio- sciences) or APC-conjugated anti-CD41 (17-0411-82, eBioscience) antibody at 1:50 dilution ratio. Propidium iodide (PI; BD Biosciences) was included to exclude the dead cells. Stable mESC lines were constructed by lentivirus transfection and sorted by GFP expression on FACSAria (BD Biosciences). Results were processed using FlowJo software (Tree Star).

Western blotting

The whole EB cell lysates were prepared using 1X Loading Buffer in combination with PhosSTOP (Roche). Samples were then denatured on a heat block at 100°C for 5 minutes. Proteins were resolved by electrophoresis on 8% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in 5% BSA (1X TBS-Tween20) and probed with the following antibodies: phosphorylated Smad1/5/8 (1:1,000; Cell Signaling), Smad1 (1:1,000; Cell Signaling), Phospho-Akt (Thr308) (1:1,000; Cell Signaling), Phospho-Akt (Ser473) (1:1,000; Cell Signaling), Akt (1:1,000; Cell Signaling), pERK1/2 (1:1,000; Cell Signaling), ERK1/2 (1:1,000; Santa Cruz), and beta-actin (1:1,000; Sigma). The membrane was detected by secondary fluorescent rabbit antibody CW800. The Western blots were quantified using ImageJ (http://imagej.nih.gov/ij/index.html).

Zebrafish maintenance and embryo production

Zebrafish maintenance, breeding, and staging were performed as described previously (Kimmel et al., 1995).

Morpholinos, siRNAs, and mRNA synthesis

Morpholino antisense oligonucleotides (MOs) were obtained from Gene Tools. siRNAs were designed and synthesized by GenePharma. The sequence of MO and siRNA is provided in Supplementary Table S2. For generating mRNA, full-length lpar1 coding sequence was cloned into pCDNA3 vector. To construct EGFP reporters, -213 to +24 bp of lpar1 gene, which contains the recognition site of Lpar1 MO, or -61 to +12 bp of atx gene, which contains the recognition site of Atx MO, was cloned upstream of EGFP gene in pcDNA3 vector, respectively. Lpar1, lpar1-EGFP, and atx-EGFP mRNA were synthesized using the mMessage mMachine kit (Ambion).

In vitro RNA synthesis and whole mount in situ hybridization

Antisense riboprobes were synthesized using the DIG RNA Labeling kit (Roche) according to the manufacturer’s instructions. WISH was performed as previously described (Kissa et al., 2008), and staining was performed with an alkaline phosphatase substrate kit (SK-5400, Vector Laboratories, Inc.). Images were captured with Olympus SZX16 microscope fitted with DP71 digital camera, 1X objective lense, and the DP controller acquisition software (Olympus, Japan).

Fluorescence microscopy

Tg(flk1:EGFP) transgenic embryos were embedded in 1.0% low melting point agarose for imaging. Images were obtained using Olympus SZX16 system.

Statistical analysis

Quantitative data were expressed as mean ± s.e.m. The statistical significance was analyzed by one-way ANOVA followed by LSD post hoc test for multiple comparisons, or by Student’s t-test for two comparisons. P-value of less than 0.05 was considered statistically significant.

Supplementary information for this article is available online: http://emboj.embopress.org

Acknowledgements

We thank Professor Mien-Chie Hung at the University of Texas MD Anderson Cancer Center for the constitutively active Akt mutant plasmid and Professor Xin Xie for providing small molecule chemicals. We are also grateful to Shunmei Xin, Shiyian Zhou, and Yi Jin for technical assistance. All experiments performed have been authorized by the institutional review board. This work was supported by Chinese Academy of Sciences (XDA01010302), the MOST (2014CB964802), Ministry of Health (2012BAI10B03), Shanghai Municipal Commission for Science and Technology (22ZR1452300), and the National Natural Science Foundation of China (31371419, 31301129).

Author contributions

HL and RY designed and performed the experiments, analyzed the data, and wrote the manuscript; BW analyzed data; GC and JD provided analytical tools; and GP supervised experimental design, data analysis, and manuscript preparation.

Conflict of interest

The authors declare that they have no conflict of interest.
References


