aggregate was DiI-negative, although some single CM-DiI$^-$/Kit$^+$ cells were observed (data not shown). It is possible that not all allantoic cells were CM-DiI-tagged and that non-tagged cells gave rise to single Kit$^+$ cells, given the technical difficulty of tagging all cells. We injected CM-DiI into the basal part of allantois, implying that HC clusters are originated from this part. It is also possible that chorionic cells per se may give rise to Kit$^+$ cells: chorion reportedly has a potential to generate myeloid and definitive erythroid cells (Corbel et al., 2007; Zeigler et al., 2006). Thus, although some HC clusters may have been derived from chorion, it is more likely that the mouse placenta does autonomously generate HSCs and that the allantois is at least a major source of placental HSCs. As shown in Fig. 4A, HC clusters first form cell aggregates. Although several reports suggest that HC clusters in the AGM region are derived from endothelial cells expressing VE-cadherin (Dzierzak and Speck, 2008), the HC clusters in the placenta probably did not originate from endothelial cells. Interestingly, Fraser et al. demonstrated that VE-cadherin is also expressed in HC clusters in the AGM region, indicating that VE-cadherin is not a specific marker of endothelial cells (Fraser et al., 2003). It may be further necessary to evaluate the origin of HC clusters both in the AGM region and the placenta in the future.

Niche regulation of placental HSCs

HSCs are regulated by niche cells surrounding HSCs. However, it remains unclear how embryonic HSCs are regulated by niche cells. In the bone marrow, expression of niche cell markers such as N-cadherin and CXCL12 enables their isolation by flow cytometry and has contributed greatly to an understanding of niche regulation (Arai and Suda, 2007; Sugiyama et al., 2006). Conversely, investigation of the placental niche has been impeded by a lack of markers for placental niche cells. To address this issue, we isolated niche cells surrounding HC clusters in placenta by LCM. Using this system, we obtained niche cells despite the lack of markers. HC clusters were found inside blood vessels, suggesting that niche cells are mostly composed of endothelial cells. In addition, we sorted out both endothelial and mesenchymal cells, and performed real-time PCR with SCF gene (Fig. 6). Our gene expression analysis revealed that SCF is predominantly expressed in niche cells, and protein expression analysis suggested that SCF is predominantly expressed in niche endothelial cells. In agreement, we found that SCF is predominantly expressed in endothelial cells, in particular cells surrounding HC clusters by immunostaining. Interestingly, SCF was expressed in clusters as well as in endothelial cells, implying an autocrine mechanism. It would be of interest to investigate whether SCF plays a role in specification as well as niche development. To understand the role of the SCF/Kit signal in regulating placental HSCs, we performed a loss-of-function experiment in vivo to inhibit SCF/Kit signaling in the mouse placenta using a WEC system with 10.25 dpc embryos – a stage suitable for manipulation. SCL is not required for HSC development once commitment to hematopoietic lineages has occurred (D'Souza et al., 2005; Mikkola et al., 2003b; Robb et al., 1995; Shivdasani et al., 1995). However, Gata2 is crucial for definitive hematopoiesis and functions in the generation and expansion of HSCs in the AGM region (Ling et al., 2004; Lugus et al., 2007; Tsai et al., 1994). Our study confirmed that expression of *Runx1*, *Myb* and *Gata2* was significantly downregulated compared with control samples in Kit loss-of-function analyses but *SCL* expression was not altered. Kit receptor activation plays a major role in regulating survival, proliferation and self-renewal of HSC phenotypes (Kent et al., 2008), but how SCF/Kit signal regulates *Runx1*, *Myb* and *Gata2* remains unclear. In addition to SCF/Kit signaling, other signals may regulate HC clusters. SCF secreted by niche cells may modulate proliferation of CD31$^+$/CD34$^+$/Kit$^+$ cells between 10.5 and 12.5 dpc, as shown in Fig. 5, although this proliferation might be due to an accumulation of the hematopoietic cells in the placental vasculature as this organ increases in size. Decrease of Ki-67 positive cells might be due to the downregulation of SCF by niche cells.

IL3 reportedly increases the number of HSCs in the AGM region (Robin et al., 2006). However, these authors demonstrated that IL3 has no effect on HSC activity in the placenta at 10.5 dpc, an observation compatible with our data showing that IL3 is not expressed in placental niche cells (Fig. 6A). Hedgehog, BMP4, bFGF and VEGF signals from the surrounding micro-environment are required for mesodermal cells to commit to hematopoietic cells (Dzierzak and Speck, 2008). In the AGM region, location plays a role in regulating HSC generation: ventral tissues induce AGM HSCs, whereas dorsal tissues suppress them (Peeters et al., 2009). Hedgehog protein(s) have been identified as positive effectors that increase the number of AGM HSCs (Peeters et al., 2009). Moreover, there is greater expansion of placental HSCs from 11.5 dpc to 12.5 dpc than of hematopoietic progenitors at this site, suggesting that other signals in the placental niche probably inhibit HSC differentiation (Gekas et al., 2005).

This is the first report to identify and examine the function of cytokine signals regulating HSCs in the mouse placenta. Our study is also evidence that LCM is a useful tool with which to study molecular mechanisms in specific cell aggregates. Recently, it was demonstrated that human placenta contains HSCs and that stromal cells (derived from human placenta) could support hematopoiesis (Robin et al., 2009). Clarifying how the niche regulates HSCs in the placenta could lead to an understanding of how to manipulate HSC generation from ES/iPS cells and, thus, be applicable to future clinical applications.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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References

Alvarez-Silva, M., Belo-Diabangouaya, P., Salaun, J. and Dieterlen-Lievre, F. (2003). Mouse placenta is a major hematopoietic organ. *Development* **130**, 5437-5444.

Arai, F. and Suda, T. (2007). Maintenance of quiescent hematopoietic stem cells in the osteoblastic niche. *Ann. NY Acad. Sci.* **1106**, 41-53.

Balazs, A. B., Fabian, A. J., Esmon, C. T. and Mulligan, R. C. (2006). Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood* **107**, 2317-2321.

Baumann, C. I., Bailey, A. S., Li, W., Ferkowicz, M. J., Yoder, M. C. and Fleming, W. H. (2004). PECAM-1 is expressed on hematopoietic stem cells throughout ontogeny and identifies a population of erythroid progenitors. *Blood* **104**, 1010-1016.

Caprioli, A., Jaffredo, T., Gautier, R., Dubourg, C. and Dieterlen-Lievre, F. (1998). Blood-borne seeding by hematopoietic and endothelial precursors from the allantois. *Proc. Natl. Acad. Sci. USA* **95**, 1641-1646.

Caprioli, A., Minko, K., Drevon, C., Eichmann, A., Dieterlen-Lievre, F. and Jaffredo, T. (2001). Hemangioblast commitment in the avian allantois: cellular and molecular aspects. *Dev. Biol.* **238**, 64-78.
Corbel, C. and Salaun, J. (2002). Alphallb integrin expression during development of the murine hemopoietic system. *Dev. Biol.* **243**, 301-311.
Corbel, C., Vaigot, P. and Salaun, J. (2005). (alpha)IIb Integrin, a novel marker for hemopoietic progenitor cells. *Int. J. Dev. Biol.* **49**, 279-284.
Corbel, C., Salaun, J., Belo-Diabangouaya, P. and Dieterlen-Lievre, F. (2007). Hematopoietic potential of the pre-fusion allantois. *Dev. Biol.* **301**, 478-488.
Cumano, A., Dieterlen-Lievre, F. and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* **86**, 907-916.
Cudennec, C. A., Thiery, J. P. and Le Douarin, N. (1981). In vitro induction of adult erythropoiesis in early mouse yolk sac. *Proc. Natl. Acad. Sci. USA* **78**, 2412-2416.
Czechowicz, A., Kraft, D., Weissman, I. L. and Bhattacharya, D. (2007). Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches. *Science* **318**, 1296-1299.
D'Souza, S. L., Elefanty, A. G. and Keller, G. (2005). SCL/Tal-1 is essential for hematopoietic commitment of the hemangioblast but not for its development. *Blood* **105**, 3862-3870.
Dancis, J., Jansen, V., Gorstein, F. and Douglas, G. W. (1968). Hematopoietic cells in mouse placenta. *Am. J. Obstet. Gynecol.* **100**, 1110-1121.
Dancis, J., Jansen, V., Brown, G. F., Gorstein, F. and Balis, M. E. (1977). Treatment of hypoplastic anemia in mice with placental transplants. *Blood* **50**, 663-670.
de Bruijn, M. F., Ma, X., Robin, C., Ottersbach, K., Sanchez, M. J. and Dzierzak, E. (2002). Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity* **16**, 673-683.
Downs, K. and Harmann, C. (1997). Developmental potency of the murine allantois. *Development* **124**, 2769-2780.
Dzierzak, E. and Speck, N. A. (2008). Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat. Immunol.* **9**, 129-136.
Ema, H. and Nakauchi, H. (2000). Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* **95**, 2284-2288.
Ferkowicz, M. J. and Yoder, M. C. (2005). Blood island formation: longstanding observations and modern interpretations. *Exp. Hematol.* **33**, 1041-1047.
Ferkowicz, M. J., Starr, M., Xie, X., Li, W., Johnson, S. A., Shelley, W. C., Morrison, P. R. and Yoder, M. C. (2003). CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development* **130**, 4393-4403.
Fraser, S. T., Ogawa, M., Yokomizo, T., Ito, Y., Nishikawa, S. and Nishikawa, S. (2003). Putative intermediate precursor between hematogenic endothelial cells and blood cells in the developing embryo. *Dev. Growth Differ.* **45**, 63-75.
Gekas, C., Dieterlen-Lievre, F., Orkin, S. H. and Mikkola, H. K. (2005). The placenta is a niche for hematopoietic stem cells. *Dev. Cell* **8**, 365-375.
Godin, I. and Cumano, A. (2002). The hare and the tortoise: an embryonic haematopoietic race. *Nat. Rev. Immunol.* **2**, 593-604.
Gomez, S. K. and Harrison, M. J. (2009). Laser microdissection and its application to analyze gene expression in arbuscular mycorrhizal symbiosis. *Pest Manage. Sci.* **65**, 504-511.
Goyama, S., Yamamoto, G., Shimabe, M., Sato, T., Ichikawa, M., Ogawa, S., Chiba, S. and Kurokawa, M. (2008). Evi-1 is a critical regulator for hematopoietic stem cells and transformed leukemic cells. *Cell Stem Cell* **3**, 207-220.
Houssaint, E. (1981). Differentiation of the mouse hepatic primordium. II. Extrinsic origin of the haemopoietic cell line. *Cell Differ.* **10**, 243-252.
Johnson, G. R. and Moore, M. A. (1975). Role of stem cell migration in initiation of mouse foetal liver haemopoiesis. *Nature* **258**, 726-728.
Kent, D., Copley, M., Benz, C., Dykstra, B., Bowie, M. and Eaves, C. (2008). Regulation of hematopoietic stem cells by the steel factor/KIT signaling pathway. *Clin. Cancer Res.* **14**, 1926-1930.
Khakoo, A. Y., Pati, S., Anderson, S. A., Reid, W., Elshal, M. F., Rovira, I. I., Nguyen, A. T., Malide, D., Combs, C. A., Hall, G. et al. (2006). Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J. Exp. Med.* **203**, 1235-1247.
Krishnamurthy, K., Wang, G., Rokhfeld, D. and Bieberich, E. (2008). Deoxycholate promotes survival of breast cancer cells by reducing the level of pro-apoptotic ceramide. *Breast Cancer Res.* **10**, R106.
Kulkeaw, K., Mizuochi, C., Horio, Y., Osumi, N., Tsuji, K. and Sugiyama, D. (2009). Application of whole mouse embryo culture system on stem cell research. *Stem Cell Rev.* **5**, 175-180.
Kumaravelu, P., Hook, L., Morrison, A. M., Ure, J., Zhao, S., Zuyev, S., Ansell, J. and Medvinsky, A. (2002). Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* **129**, 4891-4899.
Li, W., Johnson, S. A., Shelley, W. C., Ferkowicz, M., Morrison, P., Li, Y. and Yoder, M. C. (2003). Primary endothelial cells isolated from the yolk sac and para-aortic splanchnopleura support the expansion of adult marrow stem cells in vitro. *Blood* **102**, 4345-4353.
Ling, K. W., Ottersbach, K., van Hamburg, J. P., Oziemlak, A., Tsai, F. Y., Orkin, S. H., Ploemacher, R., Hendriks, R. W. and Dzierzak, E. (2004). GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. *J. Exp. Med.* **200**, 871-882.
Lugus, J. J., Chung, Y. S., Mills, J. C., Kim, S. I., Grass, J., Kyba, M., Doherty, J. M., Bresnick, E. H. and Choi, K. (2007). GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development* **134**, 393-405.
Matsubara, A., Iwama, A., Yamazaki, S., Furuta, C., Hirasawa, R., Morita, Y., Osawa, M., Motohashi, T., Eto, K., Ema, H. et al. (2005). Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development. *J. Exp. Med.* **202**, 1483-1492.
Matsuoka, S., Tsuji, K., Hisakawa, H., Xu, M., Ebihara, Y., Ishii, T., Sugiyama, D., Manabe, A., Tanaka, R., Ikeda, Y. et al. (2001). Generation of definitive hematopoietic stem cells from murine early yolk sac and paraaortic splanchnopleures by aorta-gonad-mesonephros region-derived stromal cells. *Blood* **98**, 6-12.
McGrath, K. E. and Palis, J. (2005). Hematopoiesis in the yolk sac: more than meets the eye. *Exp. Hematol.* **33**, 1021-1028.
Medvinsky, A. and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897-906.
Melchers, F. (1979). Murine embryonic B lymphocyte development in the placenta. *Nature* **277**, 219-221.
Mikkola, H. K., Fujiwara, Y., Schlaeger, T. M., Traver, D. and Orkin, S. H. (2003a). Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood* **101**, 508-516.
Mikkola, H. K., Klintman, J., Yang, H., Hock, H., Schlaeger, T. M., Fujiwara, Y. and Orkin, S. H. (2003b). Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. *Nature* **421**, 547-551.
Mitjavila-Garcia, M. T., Cailleret, M., Godin, I., Nogueira, M. M., Cohen-Solal, K., Schiavon, V., Lecluse, Y., Le Pesteur, F., Lagrue, A. H. and Vainchenker, W. (2002). Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells. *Development* **129**, 2003-2013.
Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., Pietryga, D. W., Scott, W. J., Jr and Potter, S. S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**, 677-689.
Mukouyama, Y., Chiba, N., Mucenski, M. L., Satake, M., Miyajima, A., Hara, T. and Watanabe, T. (1999). Hematopoietic cells in cultures of the murine embryonic aorta-gonad-mesonephros region are induced by c-Myb. *Curr. Biol.* **9**, 833-836.
North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., Marin-Padilla, M. and Speck, N. A. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563-2575.
North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E. and Speck, N. A. (2002). Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity* **16**, 661-672.
Ogawa, M., Nishikawa, S., Yoshinaga, K., Hayashi, S., Kunisada, T., Nakao, J., Kina, T., Sudo, T. and Kodama, H. (1993). Expression and function of c-Kit in fetal hemopoietic progenitor cells: transition from the early c-Kit-independent to the late c-Kit-dependent wave of hemopoiesis in the murine embryo. *Development* **117**, 1089-1098.
Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G. and Downing, J. R. (1996). AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321-330.
Osumi-Yamashita, N., Ninomiya, Y. and Eto, K. (1997). Mammalian craniofacial embryology in vitro. *Int. J. Dev. Biol.* **41**, 187-194.
Ottersbach, K. and Dzierzak, E. (2005). The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev. Cell* **8**, 377-387.
Palis, J., Robertson, S., Kennedy, M., Wall, C. and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073-5084.
Peeters, M., Ottersbach, K., Bollerot, K., Orelio, C., de Bruijn, M., Wijgerde, M. and Dzierzak, E. (2009). Ventral embryonic tissues and Hedgehog proteins induce early AGM hematopoietic stem cell development. *Development* **136**, 2613-2621.
Rhodes, K. E., Gekas, C., Wang, Y., Lux, C. T., Francis, C. S., Chan, D. N., Conway, S., Orkin, S. H., Yoder, M. C. and Mikkola, H. K. (2008). The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell Stem Cell* **2**, 252-263.
Robb, L., Lyons, I., Li, R., Hartley, L., Kontgen, F., Harvey, R. P., Metcalf, D. and Begley, C. G. (1995). Absence of yolk sac hematopoiesis from mice with a targeted disruption of the scl gene. *Proc. Natl. Acad. Sci. USA* **92**, 7075-7079.
Robin, C., Ottersbach, K., Durand, C., Peeters, M., Vanes, L., Tybulewicz, V. and Dzierzak, E. (2006). An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells. *Dev. Cell* **11**, 171-180.

Robin, C., Bollerot, K., Mendes, S., Haak, E., Crisan, M., Cerisoli, F., Lauw, I., Kaimakis, P., Jorna, R., Vermeulen, M. et al. (2009). Human placenta is a potent hematopoietic niche containing hematopoietic stem and progenitor cells throughout development. *Cell Stem Cell* **5**, 385-395.

Samokhvalov, I. M., Samokhvalova, N. I. and Nishikawa, S. (2007). Cell tracing shows the contribution of the yolk sac to adult haematopoiesis. *Nature* **446**, 1056-1061.

Scholzen, T. and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. *J. Cell Physiol.* **182**, 311-322.

Shivdasani, R. A., Mayer, E. L. and Orkin, S. H. (1995). Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* **373**, 432-434.

Silva, J., Dasgupta, S., Wang, G., Krishnamurthy, K., Ritter, E. and Bieberich, E. (2006). Lipids isolated from bone induce the migration of human breast cancer cells. *J. Lipid Res.* **47**, 724-733.

Sugiyama, D. and Tsuji, K. (2006). Definitive hematopoiesis from endothelial cells in the mouse embryo; a simple guide. *Trends Cardiovasc. Med.* **16**, 45-49.

Sugiyama, D., Ogawa, M., Hirose, I., Jaffredo, T., Arai, K. and Tsuji, K. (2003). Erythropoiesis from acetyl LDL incorporating endothelial cells at the preliver stage. *Blood* **101**, 4733-4738.

Sugiyama, D., Arai, K. and Tsuji, K. (2005). Definitive hematopoiesis from acetyl LDL incorporating endothelial cells in the mouse embryo. *Stem Cells Dev.* **14**, 687-696.

Sugiyama, D., Ogawa, M., Nakao, K., Osumi, N., Nishikawa, S., Arai, K., Nakahata, T. and Tsuji, K. (2007). B cell potential can be obtained from pre-circulatory yolk sac, but with low frequency. *Dev. Biol.* **301**, 53-61.

Sugiyama, T., Kohara, H., Noda, M. and Nagasawa, T. (2006). Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-988.

Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. and Orkin, S. H. (1994). An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221-226.

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A. H. and Speck, N. A. (1996). Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. USA* **93**, 3444-3449.

Watson, E. D. and Cross, J. C. (2005). Development of structures and transport functions in the mouse placenta. *Physiology (Bethesda)* **20**, 180-193.

Yoder, M. C., Hiatt, K., Dutt, P., Mukherjee, P., Bodine, D. M. and Orlic, D. (1997a). Characterization of definitive lymphohematopoietic stem cells in the day 9 murine yolk sac. *Immunity* **7**, 335-344.

Yoder, M. C., Hiatt, K. and Mukherjee, P. (1997b). In vivo repopulating hematopoietic stem cells are present in the murine yolk sac at day 9.0 postcoitus. *Proc. Natl. Acad. Sci. USA* **94**, 6776-6780.

Yuasa, H., Oike, Y., Iwama, A., Nishikata, I., Sugiyama, D., Perkins, A., Mucenski, M. L., Suda, T. and Morishita, K. (2005). Oncogenic transcription factor Evi1 regulates hematopoietic stem cell proliferation through GATA-2 expression. *EMBO J.* **24**, 1976-1987.

Zeigler, B. M., Sugiyama, D., Chen, M., Guo, Y., Downs, K. M. and Speck, N. A. (2006). The allantois and chorion, when isolated before circulation or chorio-allantoic fusion, have hematopoietic potential. *Development* **133**, 4183-4192.