E．結論

本研究では、貧血用漢方薬として代表的な人参養栄湯、十全大補湯、四物湯類の他、大防風湯を含めた計 4 種類の漢方薬に関して、その作用機序の分子生物学的解明を目的として *in vitro* および *in vivo* 解析を行った。

In vitro においては、マウス成体骨髄単核球細胞へ 4 種類の漢方薬を個別に添加後、細胞増殖数評価および細胞増殖を反映する *c-Myc* mRNA の発現解析を行った結果、人参養栄湯が有意に血液細胞増殖を亢進することが明らかとなった。他 3 種類の漢方薬に関して、少なくとも本研究で使用した培養条件では血液細胞増殖の亢進が認められなかったため、培養条件および評価細胞の選択に関しては今後の検討課題である。さらに、人参養栄湯を構成する生薬の評価を行った結果、上述と同様の解析にて黄耆が最も血液細胞増殖を亢進したが、構成生薬の効果は相助的であることが明らかとなった。

人参養栄湯は貧血治療用漢方薬としての使用実績がある背景から、赤血球造血に着目し解析を進めた結果、Ter119 マーカーを指標とした赤血球系細胞数の亢進は一部認められたものの、*Gata1* mRNA, *Klf1* mRNA 発現を指標とした赤血球造血の亢進は認められなかった。さらに解析を進めた結果、人参養栄湯は *CD45* mRNA, *Pu.1* mRNA 発現を指標とした白血球造血亢進および、*Bcl-2* mRNA 発現を指標とした抗アポトーシス亢進への関与が認められた。以上の結果より、*in vitro* 添加培養実験系では、人参養栄湯の作用機序として、赤血球造血だけでなく白血球造血に関与する evidence を分子生物学的手法により取得できた。

さらに、人参養栄湯の作用機序を詳細に検討するために、DNA マイクロアレイ法による網羅的遺伝子発現解析を行い、発現亢進および発現低下した分子の同定を行った。候補分子の検証の結果、白血球造血を亢進する *Rasgrp1* mRNA および *Dok2* mRNA の発現に関しては *in vitro* 人参養栄湯添加培養にて再現性が確認できたものの、*in vivo* 人参養栄湯投与実験後のマウス骨髄細胞における、*Rasgrp1* mRNA および *Dok2* mRNA の発現は必ずしも *in vitro* における結果と一致する結果ではなく、実験系(解析日数、培養条件、解析細胞等)の検討が必要だといえる。また、候補分子の関与する pathway を *in silico* 解析にて検討し、oxidative リン酸化をはじめ、Wnt pathway、JAK-STAT pathway、Cytokine pathway 等への

関与が示唆されたことから、今後候補分子の機能解析を行うことで、さらに人参養栄湯の作用機序の解明が期待できる。

人参養栄湯は貧血治療用漢方薬としての使用実績がある背景から、貧血マウスモデルを用いた *in vivo* 人参養栄湯投与実験を行ったところ、白血球造血のうち、特に B220 マーカーを指標とした B リンパ球造血および Mac1, F4/80 マーカーを指標とした骨髄球造血が亢進していた。以上の結果から、*in vivo* 投与実験系においても、人参養栄湯の作用機序に関する evidence を分子生物学的手法により、一部であるが取得できた。

今後の課題として、*in vivo* における evidence の集積が必要だと考えられる。人参養栄湯の遺伝子レベルでの効果の傍証を取得するために、現在、人参養栄湯添加後の miRNA 発現変化を受託にてアレイ解析中であり、人参養栄湯のサロゲートマーカー同定を予定している。また当初予定していた CAGE 解析は、サンプル量の調整と品質保持の観点で解析が困難であった。少量で解析可能な機器が開発中であり、機器開発後着手を検討している。

Ｆ．健康危険情報

国民の生命、健康に重大な影響を及ぼすと考えられる研究成果は得られていない。

G．研究発表

1.　論文発表

(原著)

1. Antas VI, Al-Drees MA, Prudence AJ, Sugiyama D and Fraser ST.
Hemogenic endothelium: a vessel for blood production.
Int J Biochem Cell Biol. 45(3):692-695, 2013.

2. Sugiyama D, Kulkeaw K, and Mizuochi C.
TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts.
Mechanisms of Development 130(2-3): 195-206, 2013.

3. Mizuochi C, Fraser ST, Biasch K, Horio Y, Kikushige Y, Tani K, Akashi K, Tavian M and Sugiyama D.
Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis.
Plos One 7(4): e35763, 2012.

4. Kulkeaw K, Inoue T, Mizuochi C, Horio Y, Ishihama Y and Sugiyama D.
Ectopic expression of Hmgn2 antagonizes mouse erythroid differentiation in vitro.
Cell Biol Int. 36(2): 195-202, 2012.

(総説)

5. Tan KS, Tamura K, Lai MI, Veerakumarasivam A, Nakanishi Y, Ogawa M, and Sugiyama D.
Molecular pathways governing development of vascular endothelial cells from ES/ iPS cells.
Stem Cell Reviews and Reports DOI 10.1007/s12015-013-9450-7, (Manuscript accepted in May 2013).

6. Lim WF, Inoue T, Tan KS, Lai MI, and Sugiyama D.

Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells.
Stem Cell Research & Therapy (Manuscript accepted in May 2013).

7. Kulkeaw K, and Sugiyama D.
Zebrafish erythropoiesis and the utility of fish as models of anemia.
Stem Cell Research & Therapy 3(6): 55, 2012.

8. Inoue-Yokoo T, Tani K, and Sugiyama D.
Mesodermal and Hematopoietic Differentiation from ES and iPS Cells.
Stem Cell Reviews and Reports DOI 10.1007/s12015-012-9388-1, 2012.

2. 学会発表
（国際学会）

1．杉山大介
Embryonic regulation of the hematopoietic stem cell niche and implications for its expansion.
The 3rd Meeting of Asian Cellular Therapy Organization.
November 15 - 17, 2012, Chiangmai (Thailand)
口頭発表（招待講演）

2. 杉山大介
Embryonic regulation of the hematopoietic niche and implications for haematotherapy.
1st International Conference on Stem Cells.
September 6-11, 2012, Chania (Greece)
口頭発表（招待講演）

3. 水落ちよ、堀尾有可、井上朋子、クンケールカセム、ワイフェンリム、カティアバッチ、トランコンホアン、タビアンマニュエラ、杉山大介
Intra-aortic clusters undergo endothelial to hematopoietic phenotypic

changes in early embryogenesis.
第10回国際幹細胞学会，横浜，2012年6月15日
ポスター発表

4. 井上朋子、水落ちよ、堀尾有可、クンケールカセム、リムワイファン、トランコンホアン、杉山大介
Igf2はマウス卵黄嚢において赤血球造血を亢進する
第10回国際幹細胞学会，横浜，2012年6月14日
ポスター発表

5. Kasem Kulkeaw, Tomoko Inoue, Chiyo Mizuochi, Yuka Horio, Wai Feng Lim, Hoan Tran Cong, Tohru Ishitani, 杉山大介
GNAT-like protein down-regulates globin synthesis by binding acetyl CoA
第10回国際幹細胞学会，横浜，2012年6月14日
ポスター発表

6. Wai Feng Lim, Tomoko Inoue, Kasem Kulkeaw, Tze Yan Lee, Chiyo Mizuochi, Yuka Horio, Hoang Tran Cong, Elizabeth George, Mei I Lai, 杉山大介
Dok2 regulates globin gene expression in mouse embryonic erythropoiesis
第10回国際幹細胞学会，横浜，2012年6月14日
Poster.

7. 杉山大介
The use of animal models in hematopoiesis research
4th Malaysian Tissue Engineering & Regenerative Medicine Scientific Meeting
June 3-4, 2012, Langkawi
口頭発表（招待講演）

（国内学会）
8. 井上朋子、水落ちよ、堀尾有可、杉山大介
Igf2はマウス卵黄嚢において赤血球産生を相乗的に亢進する
第35回日本分子生物学会，福岡，2012年12月14日

口頭発表

9. Kasem Kulkeaw, Ornathchar Poungpair, Tomoko Inoue, Kanitta Srinoun, Chiyo Mizuochi, Wai Feng Lim, Hoang Tran Cong, Sarinthip Preedagasamzin, Yuka horio, Pa-thai Yenchitsomanus, 杉山大介
Dppa3 as a novel regulator of globin gene expression in mouse fetal liver erythropoiesis
第35回日本分子生物学会，福岡，2012年12月14日
ポスター発表

10. 井上朋子、水落ちよ、堀尾有可、クンケールカセム、リムワイファン、トランコンホアン、杉山大介
Igf2はマウス卵黄嚢において赤血球造血を相乗的に亢進する
第74回日本血液学会学術集会総会，名古屋，2012年10月19日
口頭発表

11. Kasem Kulkeaw, Ornathchar Poungpair, Tomoko Inoue, Kanitta Srinoun, Chiyo Mizuochi, Wai Feng Lim, Hoang Tran Cong, Sarinthip Preedagasamzin, Yuka horio, Pa-thai Yenchitsomanus, 杉山大介
Dppa3 as a novel regulator of globin gene expression
第74回日本血液学会学術集会総会，名古屋，2012年10月19日
口頭発表

H．知的財産権の出願・登録状況
　（予定を含む。）

1. 特許取得
- 発明の名称:　アセチル化酵素競合阻害因子Gm16515の新規用途
- 代表発明者　　杉山　大介　　准教授　（　医学研究院　）
- 出願番号:　特願2012-080211
- 出願日:2012/3/30
- 特許事務所:　三枝国際特許事務所
- 担当者　　三角　可恵（　知的財産本部　）

2. 実用新案登録

特になし

3.その他

特になし

別紙４

研究成果の刊行に関する一覧表

書籍：なし

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Tan KS, Tamura K, Lai MI, Veerakumarasivam A, Nakanishi Y, Ogawa M, Sugiyama D.	Molecular pathways governing development of vascular endothelial cells from ES/ iPS cells	Stem Cell Reviews and Reports	DOI 10.1007/s12015-013-9450-7		Manuscript accepted in May 2013
Lim WF, Inoue T, Tan KS, Lai MI, Sugiyama D.	Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells	Stem Cell Research & Therapy			Manuscript accepted in May 2013
Sugiyama D, Kulkeaw K, Mizuochi C.	TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts.	Mechanisms of Development	130(2-3)	195-206	2013
Kulkeaw K, Sugiyama D.	Zebrafish erythropoiesis and the utility of fish as models of anemia.	Stem Cell Research & Therapy	3(6)	55	2012
Inoue-Yokoo T, Tani K, Sugiyama D.	Mesodermal and Hematopoietic Differentiation from ES and iPS Cells.	Stem Cell Reviews and Reports	DOI 10.1007/s12015-012-9388-1		2012
Mizuochi C, Fraser ST, Biasch K, Horio Y, Kikushige Y, Tani K, Akashi K, Tavian M, Sugiyama D.	Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis.	PLoS One	7(4)	e35763	2012
Kulkeaw K, Inoue T, Mizuochi C, Horio Y, Ishihama Y, Sugiyama D.	Ectopic expression of Hmgn2 antagonizes mouse erythroid differentiation in vitro.	Cell Biology International	36(2)	195-202	2012

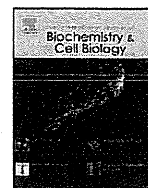
次ページより研究成果の刊行物（７点）の別刷を添付する。

The International Journal of Biochemistry & Cell Biology 45 (2013) 692–695

Contents lists available at SciVerse ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel



Cells in focus

Hemogenic endothelium: A vessel for blood production

Veronica I. Antas[a], Mohammad A. Al-Drees[a,b], Alexander J.A. Prudence[a], Daisuke Sugiyama[c], Stuart T. Fraser[a,d,*]

[a] Laboratory of Blood Cell Development, Discipline of Physiology, School of Medical Sciences, University of Sydney, Australia
[b] Laboratory of Bone Marrow and Stem Cell Processing, Department of Medical Oncology, Medical Oncology and Stem Cell Transplant Center, Al-Sabah Medical Area, Kuwait
[c] Division of Hematopoietic Stem Cells, Advanced Medical Initiatives, Department of Advanced Medical Initiatives, Kyushu University Faculty of Medical Sciences, Fukuoka 812-8582, Japan
[d] Disciplines of Anatomy and Histology, School of Medical Sciences, University of Sydney, Australia

ARTICLE INFO

Article history:
Received 14 August 2012
Received in revised form
13 November 2012
Accepted 16 December 2012
Available online 25 December 2012

Keywords:
Embryo
Hematopoietic
Hemogenic
Endothelial
Stem cells

ABSTRACT

Blood cell production, or hematopoiesis, is critical to the survival of the developing mammalian embryo. The origins of hematopoietic stem cells, capable of giving rise to all blood cell types, are being revealed. During embryogenesis, hematopoietic stem and progenitor cells are generated from a unique population of vascular endothelium termed hemogenic endothelial cells. These unusual endothelial cells are found in a restricted number of sites in the conceptus and within a narrow window of embryonic development. Loss of hemogenic endothelial cells through gene ablation leads to a lack of blood production and embryonic lethality. Here, we describe historical and recent observations exploring the biology of these intriguing endothelial cells and their roles in hematopoiesis both in the embryo and, possibly, in the adult.

Crown Copyright © 2012 Published by Elsevier Ltd. All rights reserved.

Cell facts

- Hemogenic endothelial cells are extremely rare.
- Hemogenic endothelial cells are able to differentiate into hematopoietic stem and progenitor cells.
- In the embryo, hemogenic endothelial cells are restricted to a small number of anatomical sites.
- Hemogenic endothelial cells co-express hematopoietic and endothelial genes and proteins.
- Hemogenic endothelial cells are highly conserved among vertebrates and are found in fish, reptiles, birds and mammals.

1. Introduction

Hemogenic endothelial cells (herein termed hemEC) are rare, differentiated vascular endothelial cells that generate hematopoietic (blood) cells during embryogenesis. In mammals, hematopoiesis, or blood cell production, occurs in successive waves that take place in distinct regions of the conceptus. The first transient wave of blood production is primitive hematopoiesis and is generated in the extra-embryonic yolk sac (YS). Definitive hematopoiesis commences later in development and characterised by the life-long generation of all hematopoietic lineages including hematopoietic stem cells (HSC) (extensively reviewed in Medvinsky et al., 2011).

The idea that endothelial cells in the developing embryo can generate blood cells was first postulated in the early 20th century. Pioneering microscopists observed "hematopoietic clusters" arising from the vascular endothelium of the dorsal aorta (DA) in numerous species. The origin and nature of these clusters rapidly became a controversial issue. It was argued that these clusters were not hematopoietic cells, that no evidence existed supporting an endothelial origin or that they were simply dividing cells of the vessel wall. H.E. Jordan, in 1917, feistily summarized the arguments for and against the "hemogenic capacity of endothelial cells in the dorsal aorta". A century later, molecular tools are helping to demonstrate a hemogenic endothelial origin of hematopoiesis in the embryo. While other vertebrate models such as the zebrafish have been very useful in studying hemogenic endothelial cell biology, we will restrict this review to mammals as exemplified by the mouse.

Hemogenic endothelial cells are rare cells found in restricted anatomical locations within a narrow window of development

* Corresponding author at: Laboratory of Blood Cell Development, Disciplines of Physiology, Anatomy and Histology, Room 233, Medical Foundation Building, 92-94 Parramatta Road, Camperdown, NSW 2050, Australia. Tel.: +61 2 9036 3313.
E-mail address: stuart.fraser@sydney.edu.au (S.T. Fraser).

1357-2725/$ – see front matter. Crown Copyright © 2012 Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biocel.2012.12.013

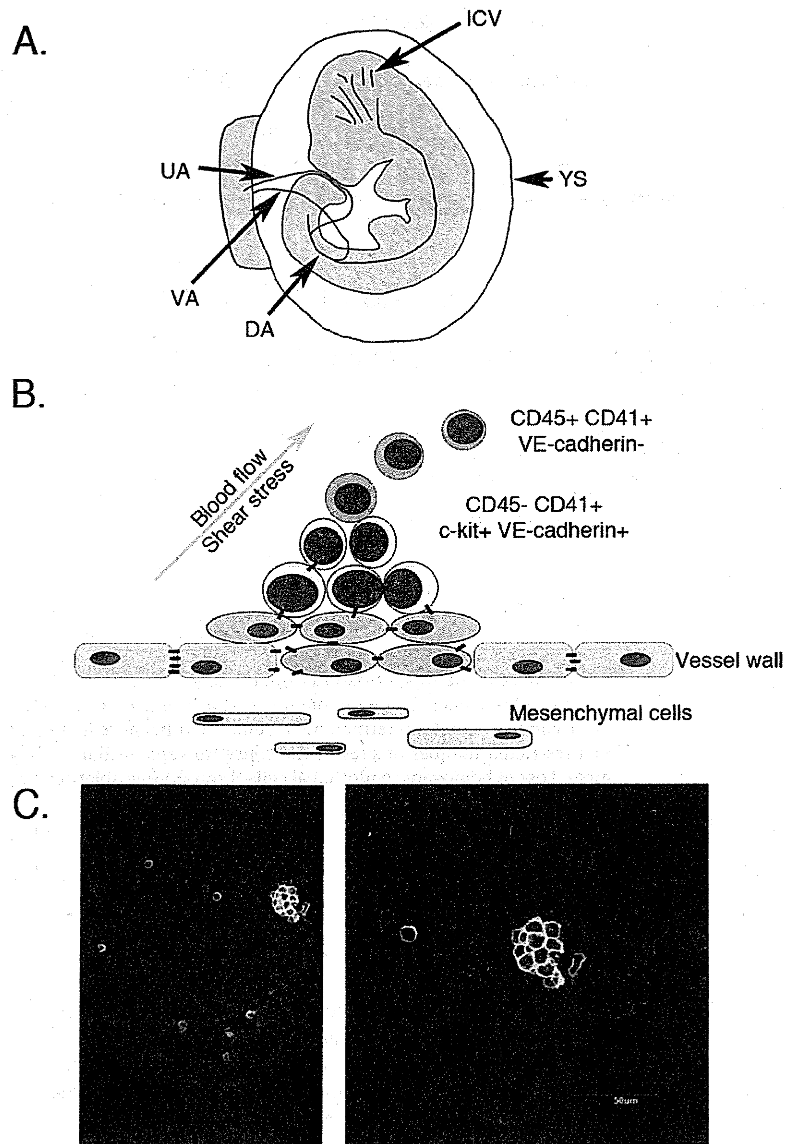


Fig. 1. The anatomy of hemogenic endothelium. (A) Hemogenic endothelial cells are found throughout the developing conceptus. A representation of a mid-gestation mou embryo shows the major anatomical location of hemogenic endothelial cells including the placenta (blue), vitelline artery (VA), umbilical artery (UA), dorsal aorta (DA), yo sac (YS) and the intracerebral vessels in the head (ICV). (B) shows a model of the developmental progression of free-floating hematopoietic cells from intra-aortic clusters. Mesenchymal cells (green) underlying the cluster are thought to transmit growth and differentiation signals. The hemogenic endothelial cells (blue) in this region loosen their adherens junctions with neighbouring endothelium (grey), down-regulate endothelial markers (yellow) and up-regulate blood cell surface antigens in response to blood flow and other signals (red). (C) Immunostaining and confocal imaging of the E10.5 AGM reveals c-Kit$^+$ clusters adherent to the vascular wall. c-Kit protein is labeled in green while nuclei are counterstained in blue with ToTo-3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Fig. 1A). The embryonic mouse tissues which possess hemogenic endothelial cells are the YS, the DA at the level of the developing gonad/mesonephros (AGM) and the placenta (Zovein et al., 2008). This unusual endothelial cell type is also found in the vitelline artery (VA) connecting the embryo and YS, and the umbilical artery (UA) linking the embryo proper and placenta (Zovein et al., 2008). Very recently and quite controversially, it has been reported that the embryonic head may also contain hemEC activity (Li et al., 2012). In these tissues, groups of hematopoietic cells extend from the vessel wall into the vascular lumen and have been termed hematopoietic, aortic, intra-aortic or intra-arterial clusters (Fig. 1B and C). In the mouse DA, hematopoietic clusters are detected from E9.5 until E14, with the greatest number occurring at E10.5 (Yokomizo and Dzierzak, 2010).

The vast majority of endothelial cells in the embryo are not hemogenic. How can we identify those that can give rise to blood cells? A single marker to segregate hemogenic from non-hemogenic endothelial cells has not yet been identified. Expression of surface antigens such as CD31 (PECAM-1), CD34 and Flk1 (VEGFR2) is shared by both endothelial and hematopoietic cells (Nishikawa et al., 1998b). Markers with greater lineage-specificity for endothelial cells such as Vascular Endothelial (VE)-cadherin have been used in combination with hematopoietic-specific antigens (CD45 and Ter-119), to isolate vascular endothelial cells (Nishikawa et al., 1998b; Fraser et al., 2002) (Fig. 1B). Further dissection of this population to enrich for hemogenic activity has included the use of; integrin α4 (CD49d) expression (Ogawa et al., 1999), Hoechst dye efflux (Nadin et al., 2003) or the

Flk1 promoter/enhancer to drive green fluorescent protein (GFP) expression (Hirai et al., 2003). Intermediate stages of development between integrated endothelial cells and free-floating blood cells have been identified according to up-regulation of c-Kit, CD41 and CD45 (for example; Boisset et al., 2010; Mizuochi et al., 2012) (Fig. 1C). Purified cells can then be tested for hemogenic potential *via in vitro* colony forming assays or transplantation studies to determine HSC activity.

2. Cell origin and plasticity

There are two main models describing the embryonic origin of hematopoietic cells. The first proposes that a mesodermal-derived precursor cell termed the hemangioblast is capable of generating both endothelial and hematopoietic cells (Huber et al., 2004). A second model argues that blood cells arise from hemEC (Nishikawa et al., 1998b). Although the two models have been seen as mutually exclusive, an *in vitro* study suggests that they may complement each other (Lancrin et al., 2009). The hemangioblast may be linked to the hematopoietic cell progeny *via* a hemogenic endothelium intermediate (Lancrin et al., 2009).

Intra-aortic clusters derived from hemEC, were initially thought to be exclusively located on the ventral aspect of the arterial wall however clusters have recently been observed on the dorsal side of this vessel (Taoudi and Medvinsky, 2007). Functional characterization of dorsal and ventral aortic clusters showed that initiation and expansion of definitive HSCs is exclusive to the ventral aspect of the DA (Taoudi and Medvinsky, 2007). In a set of technically challenging studies, the budding and release of hematopoietic cells was monitored from *ex vivo* cultured dorsal aortae. GFP driven by a Ly6C regulatory element was used to mark differentiating endothelial and hematopoietic cells. GFP^{+} cells can clearly be observed "budding off" the vascular wall of the cultured embryo slices (Boisset et al., 2010).

A broad range of soluble signaling systems have been implicated in hemEC development (recently reviewed in Kaimakis et al., 2012). The potent TGF-β family member bone morphogenic protein 4 is expressed by the mesenchyme underlying hematopoietic clusters in the mouse and human aorta. Hedgehog proteins, fibroblast growth factors and vascular endothelial growth factors (VEGF) have also been implicated in hemEC function (reviewed in Kaimakis et al., 2012). Direct cell–cell contact is also critical in hemogenic endothelial cell function. *Notch1* and its intracellular signaling adaptor protein RBPkj are essential for the development of hematopoietic cells from endothelium (Robert-Moreno et al., 2008). *Notch1* and its ligands (*Jag1*, *Jag2* and *Dll4*) are expressed in the DA and induce expression of the transcription factor *Gata2* (Robert-Moreno et al., 2008).

The transcription factor *Runx1* is critical for hemEC development. Conditional deletion of *Runx1* in endothelial cells results in a failure to proceed through the endothelial-hematopoietic transition (Chen et al., 2009). In contrast, when *Runx1* was deleted within the hematopoietic compartment, no such defect was observed (Chen et al., 2009). *Runx1* regulates the expression of crucial hematovascular transcriptional regulators such as *Scl*, *Lmo2* and *Gata2* (reviewed extensively in Swiers et al., 2010). *Scl* in turn regulates hemEC development by repressing the expression of myocardial transcriptional regulators. This was strikingly demonstrated by the abnormal presence of cells expressing cardiomyocyte proteins in the *Scl*-deficient YS (Van Handel et al., 2012). Within the embryo proper, the balance between *Runx1* and *HoxA3* expression appears to regulate the hemogenic potential of endothelial cells (Iacovino et al., 2011). The onset of hemogenic activity in the DA is marked by up-regulation of *Runx1* expression accompanied by a loss of *HoxA3* expression. *Runx1* is capable of erasing the *HoxA3*-driven endothelial program, allowing hematopoietic differentiation to occur (Iacovino et al., 2011). The Ets-like transcription factor ETV2 is expressed by hemEC and crucial in their formation (Wareing et al., 2012). The interplay between soluble signaling mediators, physiological influences and transcriptional regulation is illustrated by the requirement for nitric oxide signaling in hemEC formation. Nitric oxide, induced by the shear force of the circulation, activates *Runx1* expression in the AGM leading to hemEC formation (North et al., 2009).

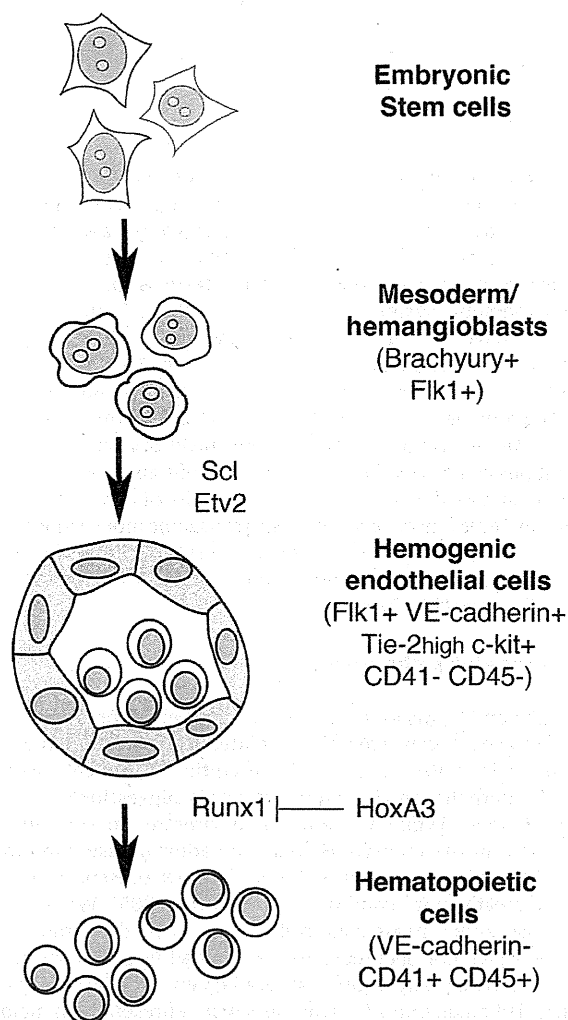


Fig. 2. The *in vitro* differentiation of embryonic stem cells into hemogenic endothelial cells. ES cell differentiation is a useful model system for dissecting the developmental stages leading from an undifferentiated progenitor to mature, functional blood cells. The expression profiles of selected transcriptional regulators and cell surface markers are shown (references included in text).

Dissecting developmental pathways in the mouse embryo is challenging due to the small size of early stage embryos along with development occurring *in utero*. The *in vitro* differentiation of embryonic stem (ES) cells has proven to be a useful model system as ES cells are easy to manipulate genetically, can be scaled-up to obtain large numbers of intermediate-stage cells (Nishikawa et al., 1998a) and can be monitored through live-cell imaging systems. Combining these attributes, the generation of free-floating hematopoietic cells from adherent endothelial cells could be observed (Lancrin et al., 2009; Eilken et al., 2009). The differentiation pathway of ES cells into hemEC is illustrated in Fig. 2. This system is particularly effective in determining the function of genes which, if deleted, lead to embryonic lethality. *In vitro* differentiation

of *Etv2*-deficient ES cells, for example, demonstrated the essential role for Etv2 in the formation of hemEC (Wareing et al., 2012).

3. Functions

The main function of hemogenic endothelium is to generate definitive hematopoietic precursors and stem cells. This has been demonstrated by flow cytometric purification and transplantation of VE-cadherin^{+}CD45^{-} cells from the embryo into newborn myeloablated mice resulting in long-term reconstitution of the hematopoietic system (Fraser et al., 2002). Transgenic mouse models have also been utilized to tag and trace the progeny of embryonic endothelial cells from the AGM, YS, UA, VA and placenta and compellingly demonstrated a vascular endothelial origin of hematopoietic cells in the adult bone marrow (Zovein et al., 2008). HemEC isolated from different stages of embryonic development possess distinct hematopoietic potentials (Fraser et al., 2002; Taoudi and Medvinsky, 2007). The diversity of hemEC function has been dissected more recently using transgenic mouse models (Chen et al., 2011). Earlier hemEC generate erythroid/myeloid progenitors whereas hemEC from the later-stage AGM region give rise to multilineage HSC (Chen et al., 2011).

4. Associated pathologies

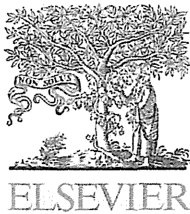
Are hemEC only found in the embryo? Cells expressing endothelial surface antigens have been isolated from the human umbilical cord at birth (Wu et al., 2007). In culture, these cells generated both endothelial and hematopoietic cells suggesting they are possibly hemEC. While VE-cadherin is present on cells with HSC potential in the embryo, HSCs in the adult mouse bone marrow lack VE-cadherin suggesting hemEC are not present in this tissue under steady-state conditions (Kim et al., 2005). Whether this is the case under stress conditions is yet to be determined. While hemEC have not been described in any adult pathological conditions it has been suggested that capillary endothelial cells in human infantile hemangioma (IH) tumours may represent hemangioblasts and/or hemEC (Itinteang et al., 2010). Proliferating IH endothelial cells in immature capillaries express hemangioblastic, endothelial markers and hematopoietic markers (Itinteang et al., 2010). Hemangioblastomas associated with von Hippel-Lindau disease develop throughout the central nervous system and express the hemangioblastic marker Brachyury as well as hematovascular proteins such Scl, Flk1 and CD133 (Park et al., 2007). Once isolated and cultured *in vitro*, cells from these hemangioblastomas could give rise to both endothelial and hematopoietic cells (Park et al., 2007). While the hemangioblast program may be re-activated during cancer progression, it remains to be determined whether the hemogenic endothelial program is also re-activated or whether this unique biological process is truly restricted to embryogenesis.

Acknowledgements

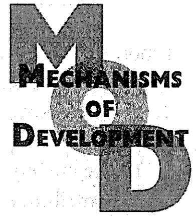
This review is a very condensed summary of a complex field and we apologise to the authors whose work we could not cite due to space restrictions. This work was supported by funding from the Sydney Medical School New Staff Grant and by the School of Medical Sciences, University of Sydney (STF). MAD is supported by the Department of Medical Oncology, Medical Oncology and Stem Cell Transplant Center, Ministry of Health, Kuwait. We thank the Research Support Center, Graduate School of Medical Sciences, Kyushu University and Miss Wai Feng Lim for technical support. We acknowledge Grant-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labor and Welfare (DS).

References

Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E, Robin C. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 2010;464(7285):116–20.
Chen MJ, De Obaldia ME, Yang Q, Yzaguirre AD, Yamda-Ingawa T, Vink CS, et al. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. Cell: Stem Cell 2011;9(6):541–52.
Chen MJ, Li Y, De Obaldia ME, Yang Q, Yzaguirre AD, Yamada-Inagawa T, et al. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. Nature 2009;457(7231):887–91.
Eilken HM, Nishikawa S-I, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. Nature 2009;457(7231):896–900.
Fraser ST, Ogawa M, Yu RT, Nishikawa S, Yoder MC, Nishikawa S. Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. Experimental Hematology 2002;30(9):1070–8.
Hirai H, Ogawa M, Suzuki N, Yamamoto M, Breier G, Mazda O, et al. Hemogenic and nonhemogenic endothelium can be distinguished by the activity of fetal liver kinase (Flk)-1 promoter/enhancer during mouse embryogenesis. Blood 2003;101(3):886–93.
Huber TL, Kouskoff V, Fehling HJ, Palis J, Keller G. Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. Nature 2004;432(7017):625–30.
Iacovino M, Chong D, Szatmari I, Hartweck L, Rux D, Caprioli A. HoxA3 is an apical regulator of haemogenic endothelium. Nature Cell Biology 2011;13(1):72–8.
Itinteang T, Tan ST, Brasch H, Day DJ. Haemogenic endothelium in infantile haemangioma. Journal of Clinical Pathology 2010;63(11):982–6.
Jordan HE. Aortic cell clusters in vertebrate embryos. Proceedings of the National Academy of Sciences of the United States of America 1917;3(3):149–56.
Kaimakis P, Crisan M, Dzierzak E. The biochemistry of hematopoietic stem cell development. Biochimica et Biophysica Acta 2012.
Kim I, Yilmaz OH, Morrison SJ. CD144 (VE-cadherin) is transiently expressed by fetal liver hematopoietic stem cells. Blood 2005;106(3):903–5.
Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V, Lacaud G. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. Nature 2009;457(7231):892–5.
Li Z, Lan Y, He W, Chen D, Wang J, Zhou F, et al. Mouse embryonic head as a site for hematopoietic stem cell development. Cell: Stem Cell 2012;11(5):663–75.
Medvinsky A, Rybtsov S, Taoudi S. Embryonic origin of the adult hematopoietic system: advances and questions. Development (Cambridge, England) 2011;138(6):1017–31.
Mizuochi C, Fraser ST, Biasch K, Horio Y, Kikushige Y, Tani K, et al. Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis. PLoS ONE 2012;7(4):e35763.
Nadin BM, Goodell MA, Hirschi KK. Phenotype and hematopoietic potential of side population cells throughout embryonic development. Blood 2003;102(7):2436–43.
Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development (Cambridge, England) 1998a;125(9):1747–57.
Nishikawa SI, Nishikawa S, Kawamoto H, Yoshida H, Kizumoto M, Kataoka H, et al. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. Immunity 1998b;8(6):761–9.
North TE, Goessling W, Peeters M, Li P, Ceol C, Lord AM. Hematopoietic stem cell development is dependent on blood flow. Cell 2009;137(4):736–48.
Ogawa M, Kizumoto M, Nishikawa S, Fujimoto T, Kodama H, Nishikawa SI. Expression of alpha4-integrin defines the earliest precursor of hematopoietic cell lineage diverged from endothelial cells. Blood 1999;93(4):1168–77.
Park DM, Zhuang Z, Chen L, Szerlip N, Maric I, Li J. von Hippel-Lindau disease-associated hemangioblastomas are derived from embryologic multipotent cells. PLoS Medicine 2007;4(2):e60.
Robert-Moreno A, Guiu J, Ruiz-Herguido C, López ME, Inglés-Esteve J, Riera L, et al. Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. The EMBO Journal 2008;27(13):1886–95.
Swiers G, de Bruijn M, Speck NA. Hematopoietic stem cell emergence in the conceptus and the role of Runx1. The International Journal of Developmental Biology 2010;54(6–7):1151–63.
Taoudi S, Medvinsky A. Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta. Proceedings of the National Academy of Sciences of the United States of America 2007;104(22):9399–403.
Van Handel B, Montel-Hagen A, Sasidharan R, Nakano H, Ferrari R, Boogerd CJ, et al. Scl represses cardiomyogenesis in prospective hemogenic endothelium and endocardium. Cell 2012;150(3):590–605.
Wareing S, Eliades A, Lacaud G, Kouskoff V. ETV2 expression marks blood and endothelium precursors, including hemogenic endothelium, at the onset of blood development. Developmental Dynamics: An Official Publication of the American Association of Anatomists 2012.
Wu X, Wu X, Lensch MW, Wylie-Sears J, Daley GQ, Bischoff J. Hemogenic endothelial progenitor cells isolated from human umbilical cord blood. Stem Cells (Dayton, Ohio) 2007;25(11):2770–6.
Yokomizo T, Dzierzak E. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. Development 2010;137(21):3651–61.
Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, et al. Fate tracing reveals the endothelial origin of hematopoietic stem cells. Cell: Stem Cell 2008;3(6):625–36.



Available at www.sciencedirect.com
SciVerse ScienceDirect
journal homepage: www.elsevier.com/locate/modo



TGF-beta-1 up-regulates extra-cellular matrix production in mouse hepatoblasts

Daisuke Sugiyama *, *Kasem Kulkeaw*, *Chiyo Mizuochi*

Division of Hematopoietic Stem Cells, Advanced Medical Initiatives, Department of Advanced Medical Initiatives, Kyushu University Faculty of Medical Sciences, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 30 January 2012
Received in revised form
9 September 2012
Accepted 15 September 2012
Available online 3 October 2012

Keywords:
TGF-beta-1
Matrix
Fetal liver
Hepatoblasts
Hematopoiesis

ABSTRACT

Fetal liver is the major embryonic hematopoietic organ and is extrinsically colonized by circulating hematopoietic stem cells (HSCs). Integrin beta-1 expression on HSCs is crucial for colonization, suggesting that interaction of Integrin beta-1 with extra-cellular matrix (ECM) factors promotes HSC adherence to fetal liver. However, little is known about how ECM production is regulated in fetal liver. Here we used flow cytometry to sort fetal liver compartments and detected ECM gene and protein expression predominantly in sorted hepatoblasts. mRNA and protein analysis suggested that TGF-beta-1 expressed by hepatoblasts, sinusoid endothelial cells and hematopoietic cells, binds to the TGF-beta receptor type-2 expressed on hepatoblasts to stimulate ECM production. Intra-cardiac injection of TGF-inhibitors into mouse embryos dramatically decreased fetal liver ECM gene expression. Taken together, our observations suggest that hepatoblasts predominantly produce ECM factors under control of TGF-beta-1 in fetal liver.

© 2012 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

In the mouse embryo, definitive hematopoiesis occurs in fetal liver, which consists of hepatoblasts expressing DLK-1 (Protein delta homolog 1), sinusoid endothelial cells expressing LYVE-1 (lymphatic vessel endothelial hyaluronic acid receptor 1) and hematopoietic cells expressing CD45 or Ter119 (Mouta Carreira et al., 2001; Tanimizu et al., 2003). In fetal liver hematopoietic stem cells (HSCs) undergo extensive self-renewal and differentiate into mature hematopoietic cells, particularly erythrocytes (Johnson and Jones, 1973; Johnson and Moore, 1975; Dzierzak et al., 1998; Ema and Nakauchi, 2000; Sugiyama and Tsuji, 2006). Both morphological observation and *in vitro* experiments suggest that fetal liver itself does not produce HSCs but is colonized by HSCs of extrinsic origin after 9.5 dpc (Johnson and Jones, 1973; Johnson and Moore, 1975; Houssaint, 1981; Cudennec et al., 1981). Previously, we demonstrated that circulating HSCs expressing c-Kit could colonize fetal liver (Sugiyama et al., 2005). Taken together, these data indicate that fetal liver provides instructive signals for HSC colonization, expansion and differentiation.

Integrin beta-1 is reportedly crucial for HSC colonization of fetal liver (Hynes and Yamada, 1982; Humphries et al., 1989; Hirsch et al., 1996; Frisch and Ruoslahti, 1997). Interaction of integrin heterodimers with extra-cellular matrix factors (ECMs) likely functions as a homing mechanism and enable HSCs and hematopoietic progenitor cells to reside in fetal liver (Patel and Lodish, 1987; Tsai et al., 1987; Long and Dixit, 1990; Williams et al., 1991; Long et al., 1992; Klein et al., 1993; Strobel et al., 1997). ECMs are produced in various cell

* Corresponding author. Address: Division of Hematopoietic Stem Cells, Advanced Medical Initiatives, Department of Advanced Medical Initiatives, Kyushu University Faculty of Medical Sciences, Station for Collaborative Research1 4F, 3-1-1 Maidashi, Higashi-Ku, Fukuoka 812-8582, Japan. Tel.: +81 92 642 6146/6210; fax: +81 92 642 6146.
E-mail address: ds-mons@yb3.so-net.ne.jp (D. Sugiyama).
0925-4773/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.mod.2012.09.003

types and interact with cytokines to allow cells to interpret cytokine signaling in a particular context (Hynes and Yamada, 1982; Humphries et al., 1989; Frisch and Ruoslahti, 1997; Taipale and Keski-Oja, 1997).

In the current study, to determine which fetal liver component contributes to ECM production, we used flow cytometry to separate cells obtained from early fetal liver into hepatoblasts, sinusoid endothelial cells and hematopoietic cells, based on expression of surface molecules, and examined ECM expression in each component by real-time PCR, Western blot analysis and immunohistochemistry. Since Transforming Growth Factor (TGF)-betas are known to potentially regulate ECM production, we treated mouse embryos with their respective inhibitors and examined ECM gene expression in fetal liver (Nakamura et al., 1992; Ziyadeh et al., 1994; Chimal-Monroy and Diaz de Leon, 1999; Laping et al., 2002; Gaggioli et al., 2005). Overall, we observe that hepatoblasts predominantly produce ECM factors under control of TGF-beta-1 in fetal liver.

2. Materials and methods

2.1. *Animals*

ICR and C57BL/6J mice were purchased from Nihon SLC (Hamamatsu, Japan) and Kyudo (Tosu, Japan), respectively. *Map2k4*$^{+/-}$ mice were provided by RIKEN BioResource Center (Tsukuba, Japan). Noon of the day of the plug was defined as 0.5 day post-coitum (dpc). Embryos at 10.25, 11.5, 12.5 and 14.5 dpc were dissected in PBS under a stereomicroscope. Animals were handled according to Guidelines for Laboratory Animals of Kyushu University. This study was approved by the Animal Care and Use Committee, Kyushu University (Approval ID: A21-068-0).

2.2. *Flow cytometry*

For hematopoietic cells, circulating blood and fetal liver cells at 11.5 and 12.5 dpc were filtered through a 40 μm nylon mesh and washed once with PBS. Cells were stained with FITC-conjugated anti-mouse CD71 (BD Biosciences, San Diego, CA) or Integrin beta-1 (CD29) (BD Biosciences), PE-conjugated anti-mouse Sca-1 (BD Biosciences), PE-Cy7-conjugated anti-mouse CD45 (eBioscience, San Diego, CA), APC-conjugated anti-mouse c-Kit (BD Biosciences) and APC-Cy7-conjugated anti-mouse Ter119 (eBioscience) Abs. Cell sorting was accomplished using a FACS Aria cell sorter (BD, Franklin Lakes, NJ). For hepatoblasts and sinusoid endothelial cells, 12.5 dpc fetal liver was digested in 1 mg/mL collagenase (Washington Biochem Co., Freehold, New Jersey) in alpha-MEM containing 10% FBS, filtered through 40-μm nylon mesh, and washed once with PBS. Cells were stained with FITC-conjugated anti-mouse DLK-1 Ab (MBL, Nagoya, Japan), PE-conjugated anti-mouse LYVE-1 Ab (MBL), PE-conjugated anti-mouse Hepatocyte growth factor (HGF) receptor Ab (eBioscience), Alexa Fluor 647-conjugated anti-mouse E-cadherin Ab (eBioscience), biotin-conjugated anti-mouse TGF-beta receptor type-2 (TGFR-2) Ab (R&D Systems, Minneapolis, MN), APC-conjugated anti-mouse CD31 Ab (Biolegend, San Diego, CA), PE-Cy7-conjugated anti-mouse CD45 Ab (eBioscience), PE-Cy7-conjugated anti-mouse Ter119 Ab (eBioscience), and APC-Cy7-conjugated streptavidin (eBioscience).

2.3. *Real-time PCR*

RNA was extracted from both sorted and unsorted fetal liver samples, and cultured cells using a RiboPure™ kit (Life Technologies, Carlsbad, CA), and mRNA was reverse transcribed using a high-capacity RNA-to-cDNA kit (Life Technologies). cDNA quality was evaluated by PCR amplification of mouse *actin, beta (Actb)*. Thirty thermal cycles were used as follows: denaturation at 95 °C for 10 s, annealing at 60 °C for 20 s, and extension at 72 °C for 20 s. Gene expression levels were measured by real-time PCR with TaqMan® Gene Expression Master Mix and StepOnePlus™ real-time PCR (Life Technologies). All probes were from TaqMan® Gene Expression Assays (Life Technologies). All samples were assayed in triplicate wells. mRNA levels were normalized to *Actb* and the relative quantity (RQ) of expression was compared with a reference sample.

2.4. *Western blot analysis*

For Western blot analysis, protein was extracted from sorted cells using Qproteome® Mammalian Protein Prep Kit (QIAGEN). Lysates of whole fetal liver, sinusoid endothelial cells, hepatoblasts and hematopoietic cells were run on 12.5% SDS–polyacrylamide gels (Ready Gels J, Bio-Rad Laboratories, Hercules, CA) concurrently with a pre-stained protein marker (Precision Plus Protein™ Standards, Bio-Rad Laboratories) using Laemmle buffer. Gels were trans-blotted onto a PVDF membrane (Immobilon®-P Transfer Membrane, Millipore Billerica, MA). The membrane was blocked in 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T) at 25 °C for 1 h, washed with washing buffer (TBS-T) and reacted with 1:5000 mouse anti-TGF-beta-1 monoclonal Ab solution (R&D systems), 1:200 anti-Fibronectin Ab (Santa Cruz Biotechnology, Santa Cruz, CA), 1:2000 anti-Vitronectin Ab (EPITOMICS, Burlingame, CA) or 1:1500 rabbit anti-Beta-actin Ab (IMGENEX, Sorrento Valley, CA) at 25 °C for 2 h. The membrane was then thoroughly washed and incubated in a solution of 1:1000 goat anti-mouse IgG-HRP conjugate (R&D systems) and 1:2500 goat anti-rabbit IgG-HRP conjugate (R&D systems) at 25 °C for 1 h. After washing, signals were visualized by soaking the membrane in substrate solution (Amersham™ ECL Plus Western Blotting Detection System, GE Healthcare, Buckinghamshire, UK). Images were captured using ChemiDoc XRS (Bio-Rad Laboratories). Data were analyzed by Quantity One ver. 4.6.7 (Bio-Rad Laboratories) and displayed as intensity per mm^2.

2.5. *Immunohistochemistry*

Injected or uninjected ICR mouse embryos were fixed in 2% paraformaldehyde in PBS overnight at 4 °C and washed in PBS three times. After 27% sucrose infusion, embryos were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen vapor phase. Frozen embryos were sectioned at 20 μm, transferred onto glass slides (Matsunami, Osaka, Japan), and dried. After blocking in 1% BSA in

PBS, sections were incubated with primary antibodies overnight at 4 °C. After washing in PBS three times, sections were incubated with secondary antibodies and TOTO-3 (Life Technologies) for nuclear staining. Anti-mouse DLK-1 Ab (MBL), anti-Fibronectin Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Vitronectin Ab (EPITOMICS, Burlingame, CA), anti-mouse CD31 Ab (BD Bioscience), anti-rat beta1-integrin Ab (BD Bioscience), anti-mouse Lyve-1 Ab (MBL), anti-mouse c-Kit Ab (R&D Systems), and anti-human SMAD family member 3 (SMAD3) phospho Ser423/Ser42 Ab (EPITOMICS) served as primary antibodies and donkey anti-rabbit IgG-Alexa555, donkey anti-goat IgG-Alexa488 and Alexa568, donkey anti-rat IgG-Alexa488 (all from Life Technologies), rabbit anti-hamster IgG-Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies. Coverslips were mounted with fluorescence mounting medium (Dako Corporation, Carpinteria, CA). Slides were observed using an FV-1000 confocal microscope (Olympus, Tokyo, Japan).

2.6. *Intra-cardiac injection*

TGF-beta receptor type-1 Kinase Inhibitor (Merck, Darmstadt, Germany) and TGF-beta receptor type-1 Kinase Inhibitor II (Merck) were dissolved in DMSO at 5 mg/mL. 0.2–0.5 μl of inhibitors, anti-TGF-beta-1 blocking antibody (MAB240, R&D systems) or mouse IgG Isotype control (MBL) were administered to 10.25 dpc ICR mouse embryos by intra-cardiac injection, as previously described (Sugiyama et al., 2003, 2005; Kulkeaw et al., 2009). As stated by the manufacture, the optimal inhibitor concentration (Primary Target IC50) is 51 nM in TGF-beta receptor type-1 Kinase Inhibitor, and 23 nM in TGF-beta receptor type-1 Kinase Inhibitor II, respectively. Embryos were isolated under a stereomicroscope (Leica Microsystems MZ6, Wetzlar, Germany) in PBS. Both the uterus and deciduo capsularis were removed and the yolk sac was cut along yolk sac arteries with care to avoid excessive hemorrhage. The amnion was opened to allow needle access to the heart. The injection needle was produced by pulling a glass capillary (Narishige GC-10, Japan) using a micropipette puller (Narishige, Tokyo, Japan). Injected embryos were immediately subjected to a mouse whole embryo culture system within 1 h of isolation.

2.7. *Mouse whole embryo culture*

Injected embryos were transferred to culture bottles containing 100% rat serum supplemented with 2 mg/ml glucose in a whole embryo culture system (Ikemoto Scientific Technology, Tokyo, Japan) and cultured for 6 or 12 h at 37 °C with a continuous supply of the gas mixture (60% O_2 and 5% CO_2 balanced with N_2) in the dark (Osumi-Yamashita et al., 1997). After whole embryo culture, embryos exhibiting no conspicuous bleeding or anomalies were analyzed for mRNA and protein expression. In addition, some fetal liver samples were isolated from embryos, pooled, filtered through a 40 μm nylon mesh, and their cell number counted.

2.8. *In vitro culture*

Hepatoblasts expressing DLK-1 were sorted from fetal liver at 12.5 dpc by flow cytometry, and 10,000 were cultured in 96-well plates with Opti-MEM (Life Technologies) containing 2% of fetal bovine serum in the presence or absence of TGF-beta-1 (10 ng/mL) (Wako, Osaka, Japan). After 6 h of culture, cells were collected and analyzed for mRNA expression.

3. Results

3.1. *Integrin expression on fetal liver hematopoietic cells*

Previously, it was reported that chimeric embryos generated with a component of *integrin beta 1 (Itgb1)*-deficient embryonic stem cells exhibited *Itgb1* null hematopoietic cells in the YS and blood, but not in fetal liver, suggesting that *Itgb1* is crucial for hematopoietic cell colonization of fetal liver (Hirsch et al., 1996). To confirm expression of Integrin beta-1 protein on hematopoietic cells, we first performed flow cytometry on blood and fetal liver cells obtained from 11.5 dpc mouse embryos. Integrin beta-1 was expressed on 94.9% of HSCs (CD45+/c-Kit+/Sca-1+) and 86.4% of erythroid cells (CD45−/Ter119+) in fetal liver, and on 96.8% of HSCs (CD45+/c-Kit+/Sca-1+) in circulation, whereas it was expressed on only 0.85% of circulating erythroid cells (CD45−/Ter119+) (Fig. 1A). Immunohistochemistry of fetal liver sections at 11.5 and 12.5 dpc showed that hematopoietic cells inside blood vessels surrounded by CD31-expressing endothelial cells did not express Integrin beta-1, while most hematopoietic cells outside blood vessels did, in agreement with flow cytometry data (Fig. 1B). To identify the alpha-chain partner of the beta-1-containing heterodimer, *integrin-alpha* expression was examined at stages ranging from HSCs to mature erythroid cells in mouse fetal liver using real-time PCR. As shown in Fig. S1, HSCs and various stages of differentiated erythroid cells were isolated by flow cytometry from fetal liver at 12.5 dpc based on expression of the cell surface markers Sca-1, c-Kit, CD71 and Ter119 (see Fig. S1 for a definition of lineage markers) (Suzuki et al., 2003; Inoue et al., 2011). When *integrin-alpha* expression was compared during erythropoiesis, *integrin alpha V (ItgaV)*, *Itga5*, *Itga6*, and *Itga9* mRNAs were predominantly expressed in HSC and hematopoietic progenitor cell fractions (Fig. 2A). No *integrin-alpha* expression was detected in the mature erythroid cell fraction (Sca-1−/c-Kit−/CD71−/Ter119+). When *integrin-alpha* expression was evaluated in each hematopoietic cell fraction, *Itga4* and *Itga6* were highly expressed in both HSC and BFU-E fractions, and *Itga4* was also expressed in relatively mature erythroid cells (Fig. 2B). Taken together, these observations imply that integrins expressed on both HSCs and hematopoietic progenitor cells interact with ECM binding partners, enabling cells to adhere properly to fetal liver.

3.2. *ECM factor expression in fetal liver*

To investigate the production of ECMs, we examined expression of several ECM genes in fetal liver at 12.5 and 14.5 dpc using real-time PCR. *Vitronectin (Vtn)* and *fibronectin1 (Fn1)* mRNAs were predominantly expressed in fetal liver (Fig. 3A). Morphological observation of fetal liver sections at 14.5 dpc revealed that hepatoblasts expressing DLK-1 were in close contact with HSCs and hematopoietic progenitor cells expressing c-Kit, implying that hepatoblasts may supply factors governing activities of HSCs and hematopoietic progeni-

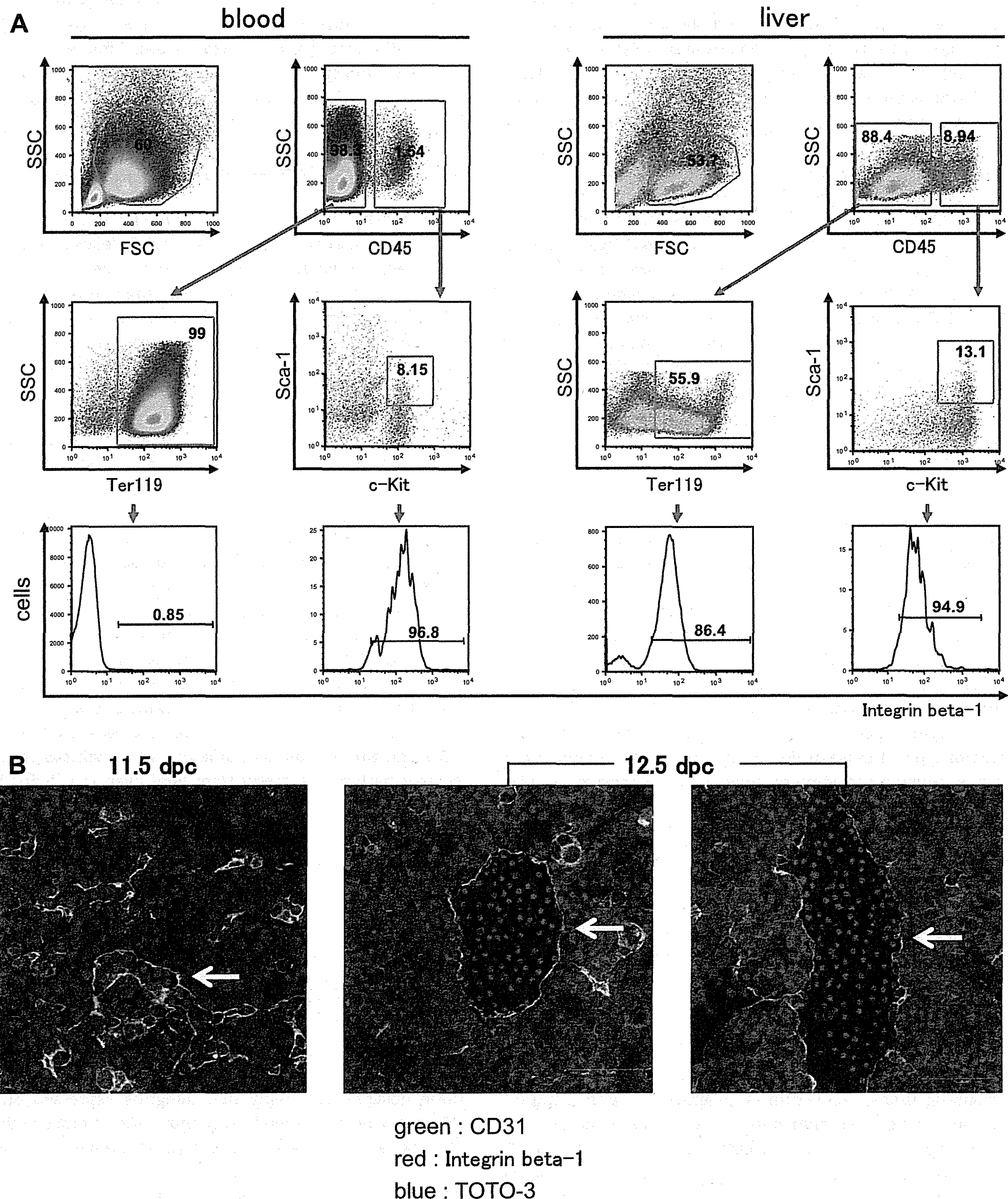


Fig. 1 – Integrin-beta-1 expression on hematopoietic cells in circulation and in fetal liver. (A) Samples were obtained from blood and fetal liver of ICR mouse embryos at 11.5 dpc. Expression of CD45 (common leukocyte antigen), Ter119 (glycophorin A), c-Kit (CD117, stem cell factor receptor), Sca-1 (stem cell antigen-1) and Integrin-beta-1 (CD29) was analyzed by flow cytometry. Integrin-beta-1 was expressed on 94.9% of HSCs (CD45+/c-Kit+/Sca-1+) and 86.4% of erythroid cells (CD45−/Ter119+) in fetal liver, and 96.8% of HSCs (CD45+/c-Kit+/Sca-1+) in circulation, whereas it was expressed on only 0.85% of erythroid cells (CD45-/Ter119+) in circulation. SSC and FSC define side scatter and forward scatter, respectively. (B) Liver sections were prepared from ICR mouse embryos at 11.5 dpc (Left) and 12.5 dpc (Middle and Right), stained with antibodies to CD31 (green) and Integrin-beta-1 (red) as well as TOTO-3 to identify nuclei, and observed under confocal microscopy. Arrows indicate that Hematopoietic cells inside blood vessels and surrounded by endothelial cells expressing CD31 do not express Integrin-beta-1, but most hematopoietic cells outside blood vessels do, in agreement with the data seen in (A). Original magnification was 20×.

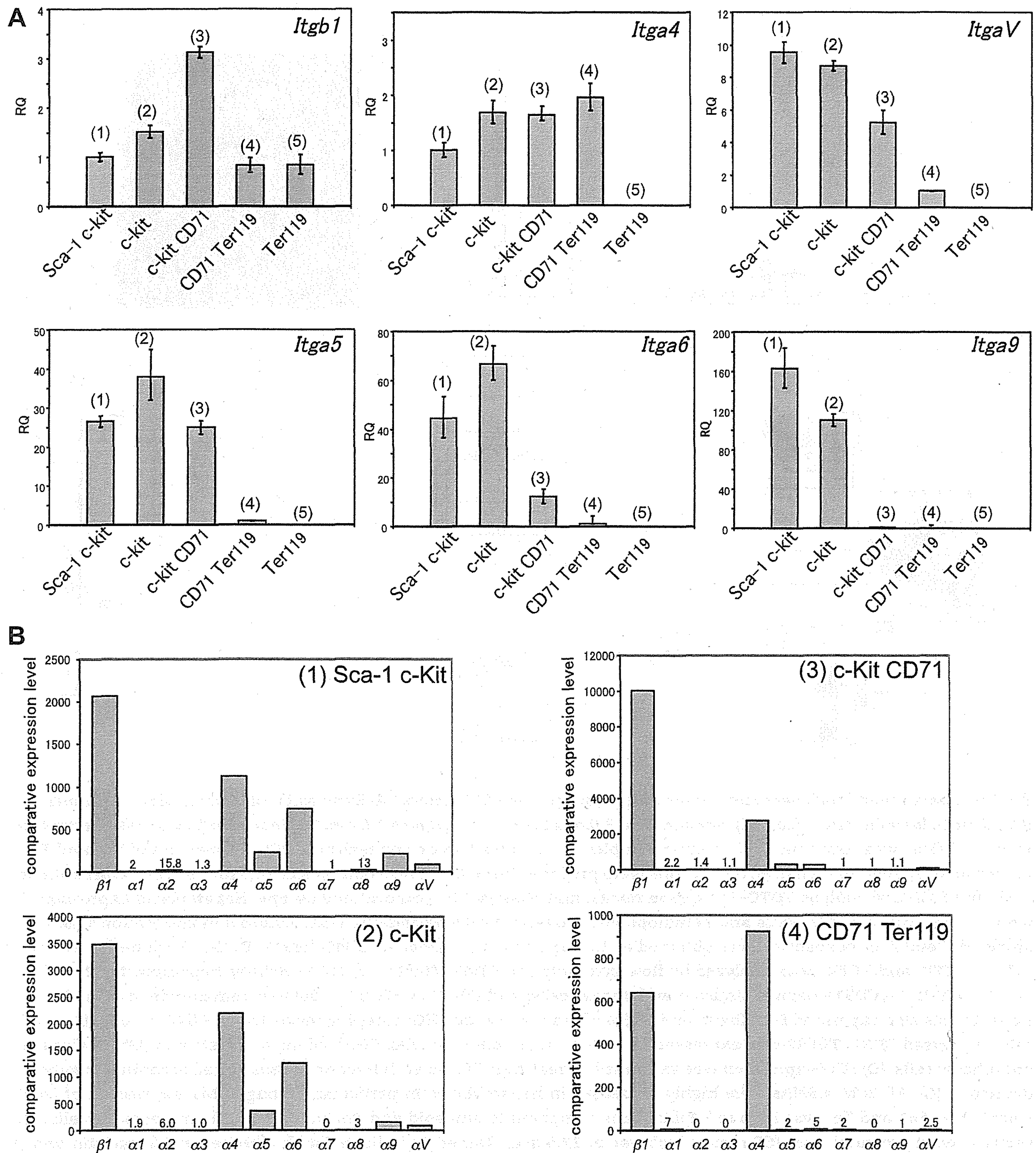


Fig. 2 – Integrin mRNA expression in fetal liver populations of the erythroid lineage. (A) HSCs and respective stages of differentiated erythroid cells were isolated from mouse fetal liver at 12.5 dpc based on expression of the cell surface molecules, Sca-1 (stem cell antigen-1), c-Kit (CD117, stem cell factor receptor), CD71 (transferrin receptor), and Ter119 (glycophorin A). Populations are defined as follows: (1) Sca-1+/c-Kit+, HSCs; (2) c-Kit+(Sca-1−/c-Kit+/CD71−/Ter119−), BFU-E; (3) c-Kit+/CD71+(Sca-1−/c-Kit+/CD71+/Ter119−), committed erythroid progenitors or CFU-E; (4) CD71+/Ter119+(Sca-1−/c-Kit−/CD71+/Ter119+), proerythroblasts; and (5) Ter119+(Sca-1−/c-Kit−/CD71−/Ter119+), mature erythroblasts and erythrocytes. Over 95% of defined HSCs expressed CD45 (common leukocyte antigen) (data not shown). Expression of *integrin-alpha* and *Itgb1* was examined in each cell fraction sorted by real-time PCR. RQ indicates relative quantification. *ItgaV*, *Itga5*, *Itga6*, and *Itga9* mRNAs were predominantly expressed in fractions (1) and (2). *Itga4* expression was not altered during erythroid cell differentiation. No expression of *integrin-alpha* was detected in fraction (5). (B) Comparison of expression levels of *integrin* genes in relatively differentiated erythroid cells is shown. Expression of *integrin-alpha* and *Itgb1* was examined by real-time PCR in each fraction of cells sorted according to gates defined in Supplementary Fig. S1. *Itga4* was expressed in fractions (3) and (4), which represent relatively mature erythroid cells.

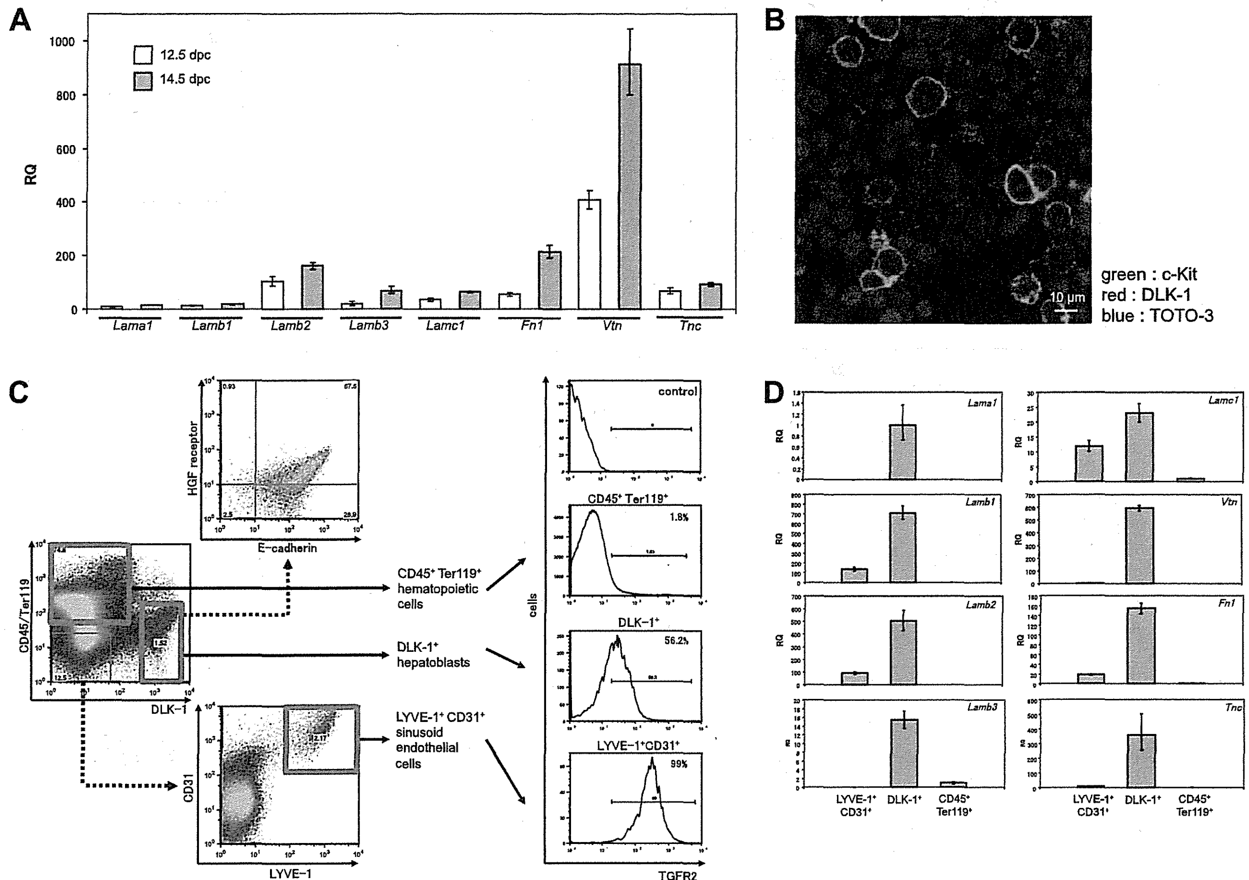


Fig. 3 – Separation of fetal liver components and expression of ECM factors. (A) Expression of *laminin, alpha 1 (Lama1), laminin B 1 (Lamb1), laminin, beta 2 (Lamb2), laminin, beta 3 (Lamb3), laminin, gamma 1 (Lamc1), fibronectin 1 (Fn1), vitronectin (Vtn)* and *tenascin C (Tnc)* was examined in fetal liver samples at 12.5 and 14.5 dpc by real-time PCR. Expression of Vtn and *Fn1* was predominantly seen in fetal liver. (B) Sections were prepared from ICR mouse embryos at 14.5 dpc, stained with antibodies to c-Kit and DLK-1, as well as TOTO-3 to define nuclei, and observed by confocal microscopy. Hepatoblasts expressing DLK-1 were in close contact with HSCs and hematopoietic progenitor cells expressing c-Kit. Original magnification was 40×. (C) A single cell fetal liver suspension was obtained at 12.5 dpc, and expression of CD45/Ter119, DLK-1, E-cadherin, HGF receptor, LYVE-1, CD31 and TGFR2 was analyzed by flow cytometry. (1) CD45−/Ter119−/DLK-1+ defines hepatoblasts; (2) CD45−/Ter119−/LYVE-1+/CD31+ defines sinusoid endothelial cells; and (3) CD45+/Ter119+ defines hematopoietic cells. 96.5% of hepatoblasts also expressed E-cadherin and 68.5% of them expressed HGF receptor, respectively. All CD45−/Ter119−/LYVE-1+ cells expressed CD31. TGFR2 was expressed in 1.8% of hematopoietic cells, 56.2% of hepatoblasts and 99% of sinusoid endothelial cells. (D) ECM expression was examined by real-time PCR in each fraction of cells sorted according to gate settings defined in (C). All ECM mRNAs were highly expressed in hepatoblasts. In particular, among ECMs, expression of *Lamb1, Lamb2, Vtn, Fn1* and *Tnc* was high in hepatoblasts compared to sinusoid endothelial cells and hematopoietic cells. (E) Liver sections were prepared from ICR mouse embryos at 12.5 dpc, stained as indicated with Fibronectin, Vitronectin and DLK-1 antibodies, as well as TOTO-3 (blue), and observed by confocal microscopy. DLK-1+ hepatoblasts expressed both Fibronectin and Vitronectin. Original magnification was 40×. (F) Vitronectin and Fibronection expression was examined by Western blot analysis in each fraction of cells sorted from ICR mouse fetal liver at 12.5 dpc, according to gate settings defined in (C). Protein expression levels were normalized to Beta-actin and displayed as intensity per mm^2. (G) Liver sections were prepared from wild-type (left panels) and *Map2k4$^{-/-}$* (right panels) mouse embryos at 12.5 dpc, stained with Fibronectin (upper; red), Vitronectin (lower; red) and TOTO-3 (blue), and observed under confocal microscopy. Expression of Fibronectin and Vitronectin proteins was down-regulated in fetal liver of *Map2k4$^{-/-}$* compared to wild-type mouse embryos. Original magnification was 20×.

tor cells (Fig. 3B). As shown in Fig. 3C, fractions of hepatoblasts, sinusoid endothelial cells and hematopoietic cells were isolated from mouse fetal liver at 12.5 dpc by flow cytometry based on the following markers: hepatoblasts, CD45−/Ter119−/DLK-1+; sinusoid endothelial cells, CD45−/Ter119−/LYVE-1+/CD31+; and hematopoietic cells, CD45+/Ter119+. We observed that 96.5% of hepatoblasts also expressed the hepatoblast marker E-cadherin. To investigate