

Brief report

Identification and characterization of a novel transcriptional target of RUNX1/AML1 at the onset of hematopoietic development

Cristina Ferreras,¹ Christophe Lancrin,^{1,2} Michael Lie-A-Ling,¹ Valerie Kouskoff,³ and Georges Lacaud¹¹Cancer Research UK Stem Cell Biology Group, Paterson Institute for Cancer Research, University of Manchester, Manchester, United Kingdom; ²European Molecular Biology Laboratory, Mouse Biology Unit, Monterotondo, Italy; and ³Cancer Research UK Stem Cell Hematopoiesis Group, Paterson Institute for Cancer Research, University of Manchester, Manchester, United Kingdom

Although the critical requirement for the transcription factor RUNX1/AML1 at the onset of hematopoietic development is well established, little is known about its transcriptional targets at this pivotal stage of blood development. Using microarrays, we identified the uncharacterized gene *A1467606* as a gene whose expression level is dramatically reduced in the absence of RUNX1. We further

demonstrated by chromatin immunoprecipitation and promoter assay a direct regulation of its transcription by RUNX1. Using a bacterial artificial chromosome transgenic approach, we established that *A1467606* is expressed during the development of the hematopoietic system in vivo and in vitro and that its expression is detected within the CD41⁺ population and marks definitive hemato-

poietic potential. Similarly, in the adult mouse, all hematopoietic cell lineages, except mature erythrocytes, express *A1467606*. Taken together, these findings indicate that *A1467606* is a novel transcriptional target of RUNX1/AML1 at the onset of hematopoietic development that is extensively expressed within the hematopoietic system. (*Blood*. 2011;118(3):594-597)

Introduction

The hemangioblast is a precursor that generates hematopoietic, endothelial, and smooth muscle lineages.^{1,2} This clonal precursor detected during embryonic stem (ES) cell differentiation and in gastrulating embryos gives rise in culture to blast colonies with both endothelial and hematopoietic potential.^{3,4} We have recently demonstrated that the generation of definitive hematopoietic cells from the hemangioblast goes through a hemogenic endothelium intermediate and that the transcription factor RUNX1 is essential for this process.⁴ Several other in vivo and in vitro studies have similarly established this generation of blood cells from a hemogenic endothelium and the requirement for RUNX1 in this process.⁵⁻¹² However, little is known about the transcriptional targets controlled by RUNX1 at this pivotal stage of blood development. In the present work, we identified *A1467606* as a novel transcriptional target of RUNX1 at the onset of hematopoietic development and demonstrated by chromatin immunoprecipitation (ChIP) and promoter assay a direct regulation of its transcription by RUNX1. We next characterized the pattern of expression of this gene in hematopoiesis in vitro using the differentiation system of ES cells as well as in vivo in embryos and adult mice. Our data demonstrate that *A1467606* is extensively expressed in hematopoietic cells and that the *A1467606*-expressing population contains all definitive hematopoietic progenitors.

previously described.¹³⁻¹⁵ Real-time polymerase chain reaction (PCR) was performed on an ABI 7900 system (Applied Biosystems) using the Roche universal probe library and primer designer (Roche Applied Science). Rabbit IgG (315-005-003 from Jackson ImmunoResearch Laboratories), Pol II (N-20; sc-899; Santa Cruz Biotechnology), and Runx1 (PC284; Calbiochem) antibodies were used for ChIP. The Dual-Luciferase Reporter Assay System (Promega) was used for luciferase assays. Time-lapse photography was performed using the IncuCyte^{FLR} live-cell imaging system (Essen Instruments) equipped with a 20× objective lens. Immunohistochemistry sections were analyzed in a Mirax Histology System (Zeiss). Slides were scanned at 200× magnification with a Zeiss Plan-Apochromat 20×/0.8 NA objective lens.

Results and discussion

Identification of a new transcriptional target of RUNX1

It is now well established that hematopoietic cells are generated from hemogenic endothelium through a RUNX1-dependent process.^{4,12} To identify transcriptional targets of RUNX1 at this early stage of hematopoiesis, we differentiated *Runx1*^{+/-} and *Runx1*^{-/-} ES cells for 3.5 days, sorted hemangioblast-enriched cell populations based on Fetal Liver Kinase-1 expression, and replated them in blast colony conditions. After 24 hours, at which time the hemogenic endothelium population is established, the transcriptome of the 2 cell lines was compared by DNA microarray analysis (supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article; <http://bioinformatics.picr.man.ac.uk/vice/PublicProjects.vice>, accession GE_CRUK/SCBR[2]). Among other genes, we identified

Methods

ES cell culture, embryoid bodies (EB) differentiation, bacterial artificial chromosome recombineering, and flow cytometry were performed as

Submitted June 30, 2010; accepted March 15, 2011. Prepublished online as *Blood* First Edition paper, April 15, 2011; DOI 10.1182/blood-2010-06-294124.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2011 by The American Society of Hematology

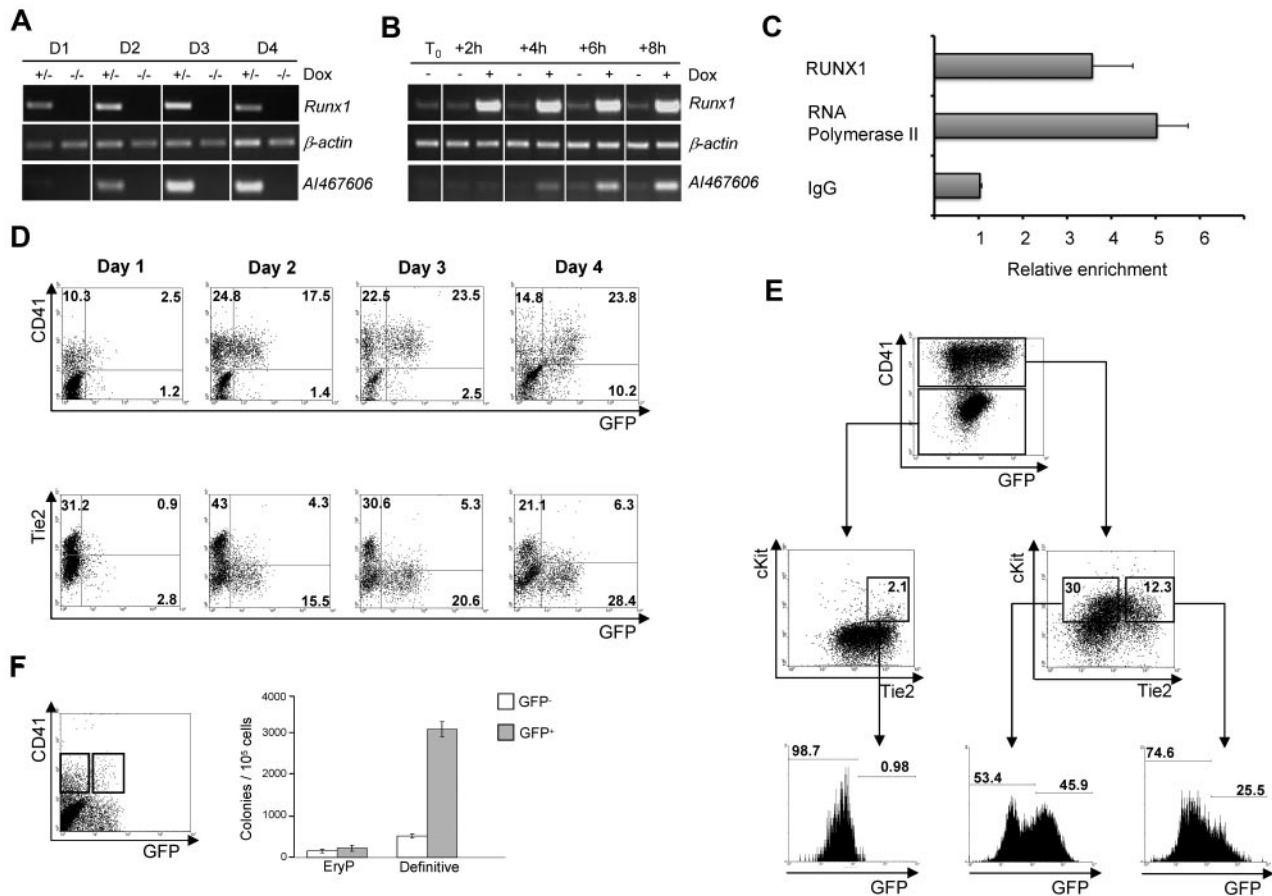


Figure 1. Identification of *AI467606* as a novel transcriptional target of RUNX1 and its expression pattern at the onset of hematopoietic development. (A) Semiquantitative reverse-transcription PCR analysis of expression of *Runx1* and *AI467606* during the course of 4 days of *Runx1*^{+/-} and *Runx1*^{-/-} blast development. (B) Semiquantitative reverse-transcription PCR analysis of expression of *Runx1* and *AI467606* after *Runx1* induction in *Runx1*^{-/-} ES cell. (C) ChIP analysis of day 2 blast colony cells. The precipitated chromatin was analyzed using primers specific for *AI467606* promoter. All values are relative to the IgG value. (D-E) FACS analysis of *AI467606*-GFP, CD41, and Tie2 coexpression in blast colonies from day 1 to day 4. Numbers indicate percentages of respective populations. (F) Number of hematopoietic colonies generated by CD41⁺GFP⁻ (white boxes) and CD41⁺GFP⁺ (gray boxes) cell populations. Definitive colony counts include macrophages, macrophages/erythrocytes, granulocytes-macrophages, and mix granulocyte-macrophage-erythroid colonies. EryP indicates primitive erythrocytes. Mean numbers of colonies from 3 dishes of a representative experiment are shown. Error bars represent SEM (n = 3).

AI467606 as a gene with lower expression level in the absence of RUNX1 (supplemental Figure 1B). The *AI467606* gene encodes for a protein of 225 amino acids with no known function. To confirm the DNA microarray results, we performed semiquantitative reverse-transcription PCR analysis comparing *Runx1*^{+/-} and *Runx1*^{-/-} cells over the 4-day time course of blast colony development. In the presence of RUNX1, the expression of *AI467606* was weak at day 1, readily detectable by day 2 of culture, and further increased over time. In contrast, in the absence of RUNX1, *AI467606* expression was barely detectable (Figure 1A). We further investigated the influence of RUNX1 on *AI467606* transcription using a *Runx1*^{-/-} ES cell line in which *Runx1* expression is induced on the addition of doxycycline.⁴ After 2 days of blast culture, doxycycline was added to the media and the cells were harvested for RNA analysis every 2 hours. *AI467606* transcription was quickly up-regulated within 4 hours after the induction of *Runx1* expression (Figure 1B), suggesting that *AI467606* is a direct RUNX1 transcriptional target. Subsequent ChIP experiments demonstrated binding of RUNX1 to the *AI467606* promoter during blast colony development (Figure 1C). Within a small 84-bp region of the *AI467606* promoter, we found 3 RUNX1-binding sites that are conserved in rat, human, chimpanzee, and mouse (supplemental Figure 1C). Both deletion of this 84-bp sequence and specific mutation of the 3 *Runx1* binding sites

resulted in a net decrease of RUNX1-induced transcription in luciferase assays (supplemental Figure 1D). Overall, these results indicate that *AI467606* is a direct transcriptional target of RUNX1 at the onset of hematopoietic development.

***AI467606* is expressed in CD41⁺ cells during blast colony formation**

To track and analyze *AI467606* expression at the single-cell level, we engineered a bacterial artificial chromosome reporter construct by replacing the coding sequence of *AI467606* with a green fluorescent protein (GFP)-neomycin cassette and established transgenic *AI467606*-GFP ES cell lines. The expression of GFP was detected in a large fraction of these ES cells (supplemental Figure 2A left) and correlated with endogenous *AI467606* transcripts (supplemental Figure 2A middle and right). We next investigated *AI467606* expression pattern during blast colony development. Consistent with our PCR results (Figure 1A), GFP expression was detected by fluorescence-activated cell sorter (FACS) from day 1 of culture onward (Figure 1D) and was morphologically associated with the appearance of round cells emerging from clusters of adherent cells (supplemental Figure 2B). GFP expression was correlated with the expression of CD41, the first marker expressed by emerging hematopoietic cells,¹⁶⁻¹⁹

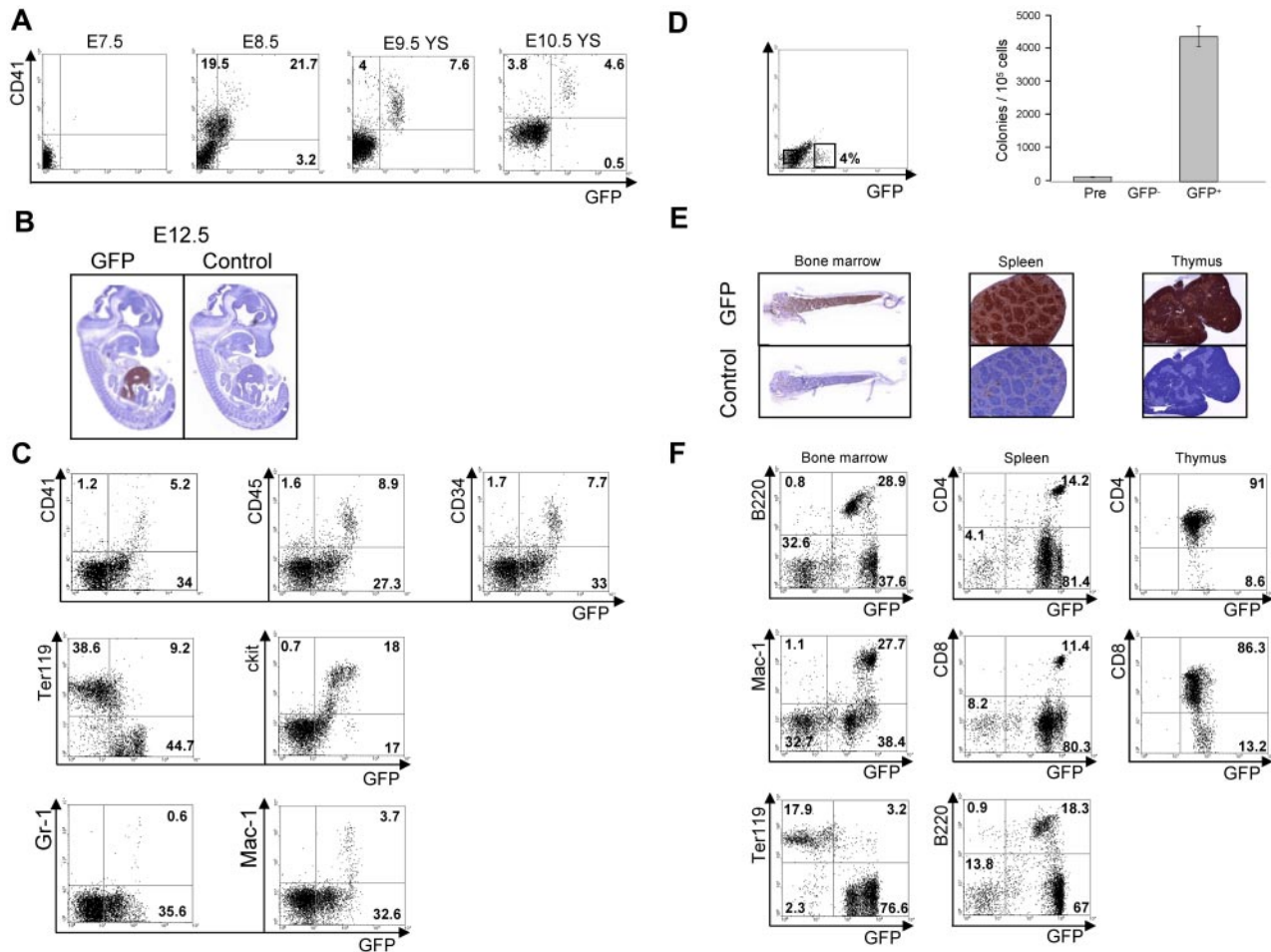


Figure 2. Analysis of the expression of *AI467606* in mouse embryos and adult hematopoietic organs. (A) FACS analysis of CD41 and *AI467606*-GFP coexpression in single embryos at E7.5, E8.5, yolk sac at E9.5 and E10.5. (B) GFP and control antibody immunohistochemistry of paraffin sections of transgenic embryos at E12.5. (C) FACS analysis of *AI467606*-GFP coexpression with CD41, CD45, CD34, Ter119, cKit, Gr-1, and Mac-1 in E12.5 fetal liver. Numbers indicate percentages of respective populations. (D) GFP FACS profile of E8.5 embryos and FACS sorting gates. Number of colonies generated by each cell population in methylcellulose. Mean numbers of colonies from 3 dishes of a representative experiment are shown. Error bars represent SEM ($n = 3$). Pre corresponds to the total population (Pre-sort). (E) GFP and control antibody immunohistochemistry of paraffin sections of bone marrow, spleen, and thymus of transgenic embryos. (F) FACS analysis of *AI467606*-GFP coexpression with B220 B-cell marker, Mac-1 myeloid marker, Ter119 erythroid marker, and CD4 and CD8 T-cell markers in bone marrow (left), spleen (middle), and thymus (right) of *AI467606*-GFP mice. Numbers indicate percentages of respective populations.

whereas fewer emerging *AI467606*-GFP⁺ cells coexpressed the endothelial marker Tie-2²⁰ (Figure 1D). We have recently shown that Tie2⁺cKit⁺CD41⁻ hemogenic endothelium cells generate hematopoietic cells through the acquisition of CD41 expression followed by the down-regulated expression of endothelial markers, such as Tie2. The proximal *Runx1* promoter is active in the Tie2⁺cKit⁺CD41⁻ hemogenic endothelium intermediate population, whereas the distal *Runx1* promoter becomes active in cells expressing CD41 and having a low Tie2 expression level.²¹ When we analyzed the expression of *AI467606*, we observed that it was barely detectable within the Tie2⁺cKit⁺CD41⁻ hemogenic endothelium population (Figure 1E), whereas within the subsequent stages of maturation *AI467606*-GFP was detected in 25.5% of the Tie2⁺cKit⁺CD41⁺ population and 46% of the Tie2^{low}cKit⁺CD41⁺ cells (Figure 1E). The absence of *AI467606* expression in hemogenic endothelium suggests that the activity of the proximal RUNX1 alone is not sufficient to drive *AI467606* expression. Altogether, these results indicate that the onset of *AI467606* expression is observed within CD41⁺ cells. As the expression of CD41 is correlated with the acquisition of hematopoietic potential, we next assessed whether the expression of *AI467606* within the CD41⁺ fraction defines a subpopulation of cells with enriched hemato-

poietic potential. GFP⁺ and GFP⁻ cells from the CD41⁺ cell population were sorted from day 5 EBs and tested in clonogenic replating assay for hematopoietic precursors. Primitive erythrocytes and definitive colonies were generated from both populations, but the double-positive population was highly enriched in definitive hematopoietic precursors (Figure 1F). Together, these observations indicate that *AI467606* expression by CD41⁺ cells marks a subpopulation of cells highly enriched in definitive hematopoietic progenitors.

AI467606 expression in vivo

We next analyzed *AI467606* expression in vivo by establishing an *AI467606*-GFP bacterial artificial chromosome transgenic mouse line. GFP⁺ cells were first detected at embryonic stage E8.5 to E10.5 within the CD41⁺ population in agreement with the data obtained in vitro (Figure 2A). Immunostaining of sections of whole embryos indicated a strong specific staining in fetal liver at E12.5 and fetal liver and thymus at E14.5 (Figure 2B; supplemental Figure 3). Flow cytometric analysis revealed that all CD41⁺, CD34⁺, cKit⁺, CD45⁺, Gr-1⁺, Mac-1⁺ cells were detected within an *AI467606*-GFP^{high} fraction. In contrast, the *AI467606*-GFP^{low}

population was mainly negative for these markers, with the exception of cKit and Ter119 (Figure 2C). These results suggest that *AI467606* is uniformly expressed in hematopoietic precursors but only weakly expressed in early erythroid cells and not expressed at later stages of erythropoiesis, a pattern reminiscent of *Runx1* expression.²²⁻²⁴ To investigate the hematopoietic potential of the *AI467606*-GFP⁺ cells, *AI467606*-GFP⁻ and GFP⁺ subpopulations isolated from GFP⁺ embryos at E8.5 were tested in a clonogenic assay. We observed that all the hematopoietic potential resides in the *AI467606*-GFP⁺ population (Figure 2D). Similar results were obtained for E11.5 fetal liver and yolk sac and E14.5 fetal liver (data not shown). These results indicate that the onset of expression of *AI467606* takes place at the early stages of hematopoietic development in CD41⁺ cells and that *AI467606* expression is sustained in hematopoietic cells in the liver. In adult mice, immunostaining indicated that *AI467606* is widely expressed in bone marrow, spleen, and thymus (Figure 2E). In the bone marrow, all cells positive for the B-cell markers B220, IgM, and IgD and the myeloid markers Gr-1 and Mac-1 coexpressed *AI467606*, whereas in contrast erythroid Ter119⁺ cells were mostly *AI467606*-GFP⁻ (Figure 2F; supplemental Figure 4A). Similarly, in spleen and thymus, most of the cells expressed *AI467606* (Figure 2F; supplemental Figure 4B-C).

Overall, these findings indicate a pan-hematopoietic pattern of *AI467606* expression both in embryonic, fetal and adult hematopoiesis. *AI467606* expression appears at the onset of hematopoiesis at E8.5 within a CD41⁺ population enriched in definitive hematopoietic progenitors and is maintained in fetal liver and adult hematopoietic cells, in contrast to CD41, which becomes more restricted to megakaryocytes at these stages. The next steps will be

to investigate the function of *AI467606* in the hematopoietic compartment. We have so far attempted knockdown with several shRNA but did not observe any striking hematopoietic phenotype. These negative results are however not fully conclusive as none of our shRNA results in a complete shutdown of *AI467606* expression, and we cannot therefore exclude that the remaining expression is enough to support hematopoietic differentiation. A definitive evaluation of the functionality of *AI467606* expression in the development of the hematopoietic system will require the generation of a knockout mouse model.

Acknowledgments

The authors thank laboratory members for critical reading of the manuscript.

This work was supported by Cancer Research UK and BBSRC.

Authorship

Contribution: C.F., C.L., and M.L. designed and performed the research, analyzed the data, and wrote the manuscript; and V.K. and G.L. designed and supervised the research project, analyzed the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Georges Lacaud, Paterson Institute for Cancer Research, University of Manchester, Wilmslow Rd, M20 4BX Manchester, United Kingdom; e-mail: glacaud@picr.man.ac.uk.

References

- Murray PDF. The development in vitro of the blood of the early chick embryo. *Proc R Soc Lond*. 1932;11:497-521.
- Sabin FR. Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of the chick during the second day of incubation: contributions to embryology. *Contrib Embryol*. 1920;9:213-262.
- Choi K, Kennedy M, Kazarov A, Papadimitriou JC, Keller G. A common precursor for hematopoietic and endothelial cells. *Development*. 1998;125(4):725-732.
- 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):892-895.
- North TE, Gu T-L, Stacy T, et al. *Cbfa2* is required for the formation of intra-aortic hematopoietic clusters. *Development*. 1999;126(11):2563-2575.
- Yokomizo T, Ogawa M, Osato M, et al. Requirement of Runx1/AML1/PEBP2B for the generation of haematopoietic cells from endothelial cells. *Genes Cells*. 2001;6(1):13-23.
- Zovein AC, Hofmann JJ, Lynch M, et al. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell*. 2008;3(6):625-636.
- Eilken HM, Nishikawa S, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature*. 2009;457(7231):896-900.
- Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E, Speck NA. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature*. 2009;457(7231):887-891.
- Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY, Traver D. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*. 2010;464(7285):108-111.
- 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):116-120.
- Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*. 2010;464(7285):112-115.
- Sroczynska P, Lancrin C, Pearson S, Kouskoff V, Lacaud G. In vitro differentiation of mouse embryonic stem cells as a model of early hematopoietic development. *Methods Mol Biol*. 2009;538:317-334.
- Gandillet A, Serrano AG, Pearson S, Lie-A-Ling M, Lacaud G, Kouskoff V. Sox7-sustained expression alters the balance between proliferation and differentiation of hematopoietic progenitors at the onset of blood specification. *Blood*. 2009;114(23):4813-4822.
- Lee EC, Yu D, Martinez de Velasco J, et al. A highly efficient Escherichia coli-based chromosome engineering system adapted for recombinogenic targeting and subcloning of BAC DNA. *Genomics*. 2001;73(1):56-65.
- Mitjavila-Garcia MT, Cailleret M, Godin I, et al. Expression of CD41 on hematopoietic progenitors derived from embryonic hematopoietic cells. *Development*. 2002;129(8):2003-2013.
- Mikkola HK, Fujiwara Y, Schlaeger TM, Traver D, Orkin SH. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood*. 2003;101(2):508-516.
- Ferkowicz MJ, Starr M, Xie X, et al. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development*. 2003;130(18):4393-4403.
- Emambokus NR, Frampton J. The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity*. 2003;19(1):33-45.
- Dumont DJ, Yamaguchi TP, Conlon RA, Rossant J, Breitman ML. tek, a novel tyrosine kinase gene located on mouse chromosome 4, is expressed in endothelial cells and their presumptive precursors. *Oncogene*. 1992;7(8):1471-1480.
- Sroczynska P, Lancrin C, Kouskoff V, Lacaud G. The differential activities of Runx1 promoters define milestones during embryonic hematopoiesis. *Blood*. 2009;114(26):5279-5289.
- Lorsbach RB, Moore J, Ang SO, Sun W, Lenny N, Downing JR. Role of RUNX1 in adult hematopoiesis: analysis of RUNX1-IRES-GFP knock-in mice reveals differential lineage expression. *Blood*. 2004;103(7):2522-2529.
- North TE, Stacy T, Mathery CJ, Speck NA, de Bruijn MF. Runx1 is expressed in adult mouse hematopoietic stem cells and differentiating myeloid and lymphoid cells, but not in maturing erythroid cells. *Stem Cells*. 2004;22(2):158-168.
- Lacaud G, Gore L, Kennedy M, et al. Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro. *Blood*. 2002;100(2):458-466.