The SHB Adapter Protein Is Required for Normal Maturation of Mesoderm during *in Vitro* Differentiation of Embryonic Stem Cells*[^1]^

Received for publication, April 28, 2006, and in revised form, September 11, 2006. Published, JBC Papers in Press, September 12, 2006. DOI 10.1074/jbc.M604084200

Vitezslav Kriz[^1], Nina Agren[^1], Cecilia K. Lindholm[^1], Samuel Lenell[^2], Johan Saldeen[^3], Jaroslav Mares[^4], and Michael Welsh[^1][2]

From the[^4] Department of Medical Cell Biology, Uppsala University, Uppsala 75123, Sweden,[^5] Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska University Hospital, Huddinge 14186, Sweden, and[^6] Institute of Biology and Medical Genetics, Charles University, Prague, 15006, Czech Republic

Definitive mesoderm arises from a bipotent mesendodermal population, and to study processes controlling its development at this stage, embryonic stem (ES) cells can be employed. SHB (Src homology 2 protein in β-cells) is an adapter protein previously found to be involved in ES cell differentiation to mesoderm. To further study the role of SHB in this context, we have established ES cell lines deficient for one (SHB*+/−*) or both SHB alleles (SHB*−/−*). Differentiating embryoid bodies (EBs) derived from these ES cell lines were used for gene expression analysis. Alternatively, EBs were stained for the blood vessel marker CD31. For hematopoietic differentiation, EBs were differentiated in methylcellulose. SHB*−/−* EBs exhibited delayed down-regulation of the early mesodermal marker Brachyury. Later mesodermal markers relatively specific for the hematopoietic, vascular, and cardiac lineages were expressed at lower levels on day 6 or 8 of differentiation in EBs lacking SHB. The expression of vascular endothelial growth factor receptor-2 and fibroblast growth factor receptor-1 was also reduced in SHB*−/−* EBs. SHB*−/−* EBs demonstrated impaired blood vessel formation after vascular endothelial growth factor stimulation. In addition, the SHB*−/−* ES cells formed fewer blood cell colonies than SHB*+/+* ES cells. It is concluded that SHB is required for appropriate hematopoietic and vascular differentiation and that delayed down-regulation of Brachyury expression may play a role in this context.

---

[^1]: This work was supported by the Swedish Research Council (31X-10822), the Swedish Cancer Foundation, The Juvenile Diabetes Research Foundation International, the Swedish Diabetes Foundation, and the Family Emfors Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[^2]: Supported by a grant from Henning and Gösta Ankarstrand and by a grant from Erland Wessler.

[^3]: To whom correspondence should be addressed: Dept. of Medical Cell Biology, P.O. Box 571, Husargatan 3, SE-751 23, Uppsala, Sweden. Tel.: 46-184714447; Fax: 46-184714059; E-mail: michael.welsh@mcb.uu.se.

[^4]: ‡ The online version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Figs. 1 and 2.

[^5]: § Supported by a grant from Henning and Gösta Ankarstrand and by a grant from Erland Wessler.

[^6]: || To whom correspondence should be addressed: Dept. of Medical Cell Biology, P.O. Box 571, Husargatan 3, SE-751 23, Uppsala, Sweden. Tel.: 46-184714447; Fax: 46-184714059; E-mail: michael.welsh@mcb.uu.se.

[^1]: The abbreviations used are: ES, embryonic stem; EB, embryoid body; VEGFR, vascular endothelial growth factor receptor; FGF, fibroblast growth factor receptor; TBS, Tris-buffered saline; SH2, Src homology 2; HNF, hepatocyte nuclear factor; RT, reverse transcription.
SHB and Embryonic Stem Cell Differentiation to Mesoderm

(21), and platelet-derived growth factor receptor (15). The R522K mutation in the SHB SH2 domain renders it inactive. The other SHB domains bind to different cytosolic proteins and thus transduce signals downstream of the receptors. SHB is implicated in many cellular processes such as differentiation of endothelial cells (22) and T cell signaling after CD3 stimulation (21). Moreover, SHB is involved in apoptotic signaling in fibroblasts, islets of Langerhans, and endothelial cells (23–25).

EBs expressing the R522K SHB mutant displayed a lower mRNA content of several liver and pancreatic markers (26). In addition, SHB was found to be essential for EB blood vessel formation (27), as EBs expressing R522K SHB failed to form normal blood vessels.

To address the role of the SHB adapter protein in development, we have established knock-out ES cell lines deficient for one or both SHB alleles. These ES cell lines were differentiated in vitro and aimed at understanding the impact of SHB on hemangiogenesis. The data suggest an important involvement of SHB in the development of mesoderm.

MATERIALS AND METHODS

Gene Targeting—The murine embryonic stem cell line GSI-1 derived from 129Sv) mice was inactivated at both (SHB−/−) or one (SHB+/−) of the SHB alleles. These were inactivated in two subsequent steps. The first targeting vector contained three loxP sites. The first and the second loxP sites flanked the neomycin resistance gene, and the second and third loxP sites bordered the first exon (Fig. 1A). One of the clones in which homologous recombination had not occurred was used as an SHB+/− control. The clone in which homologous recombination had taken place and all three loxP sites were present (loxPSHB) was treated with Cre recombinase by in vitro transfection. One of the clones, which had lost both the neomycin resistance gene and the first SHB exon, was used as a maternal clone for the second transfection.

The second targeting vector contained an insertion of the neomycin gene into the first exon without loxP sites (Fig. 1A). Two sister clones to SHB−/−, in which homologous recombination had not occurred were used as SHB+/−. Clones were screened by PCR with one primer outside the sequence of the construct (supplemental Fig. 1).

ES Cell Culture—ES cells were cultured in the presence of murine embryonic fibroblasts in Dulbecco’s modified Eagle’s medium/glutamax (Invitrogen), penicillin/streptomycin, 15% fetal bovine serum (Invitrogen), 1× nonessential amino acids (Invitrogen), sodium pyruvate (Invitrogen), monothioglycerol (Sigma), and recombinant leukemia inhibitory factor (Chemicon International, Temecula, CA) as described previously (26). Cells were grown at 37 °C in 5% CO2. For ES cell differentiation, fibroblasts and leukemia inhibitory factor were omitted. ES cells were trypsinized (5 min at 37 °C) and aggregated by the hanging drop technique (1200 cells/20 μl of medium for 2 days (28)) to form EBs. These were grown in suspension for one more day and then plated on adherent tissue culture dishes. EBs were harvested 4, 6, 8, 12, and 21 days after the initiation of differentiation. Alternatively, EBs (after formation by the hanging drop technique for 2 days) followed by 2 days of suspension culture) were grown as attachment cultures in glass chambers for an additional 7 days (yielding a total differentiation time of 11 days) and then stained for CD31 or CD41 to identify vascular structures or CD41-positive cells.

Real-time Reverse Transcription (RT)-PCR Analysis—Total RNA was isolated using the RNAeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Gene expression was quantified by real-time RT-PCR using a one-step reaction using the SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). PCR conditions were 50 °C for 20 min and 95 °C for 15 min followed by 45 cycles at 94 °C for 15 s and annealing for 25 s at the various temperatures indicated followed by extension at 72 °C for 15 s.

Alternatively, gene expression was quantified by a two-step reaction. M-MuLV reverse transcriptase (Finzymes, Helsinki, Finland) was used to convert 1 μg of total RNA into cDNA. For PCR reactions, the SYBR Green Taq Ready Mix™ (Sigma) was used. PCR conditions were initial denaturation (94 °C for 30 s) followed by 45 cycles at 94 °C for 10 s, and annealing for 25 s at the various temperatures indicated followed by extension at 72 °C for 15 s. Primer sequences are shown in supplemental Table 1. The PCR reactions were run on a Light Cycler instrument (Roche Diagnostics). Transcription levels were normalized against β-actin.

Immunohistochemical Staining of EBs with CD31—Blood vessels were stained according to a protocol described previously (29). Briefly, EBs grown on glass chambers were washed with Tris-buffered saline (TBS) and fixed with 200 mM Tris, pH 6.6, 150 mM NaCl, 37 mM ZnCl2, 3 mM zinc-acetate, 3.2 mM calcium-acetate, and 0.2% Triton X-100 (4 °C overnight). On the next day, EBs were washed with TBS and treated with 3% H2O2 in methanol to block endogenous peroxidases. EBs were washed with TBS, and unspecific interactions were blocked in 0.1M Tris-HCl/0.15M NaCl/blocking buffer. The tissue was incubated with primary antibody, rat anti-mouse CD31 (BD Biosciences) at 1/1000, washed with TBS-Tween, and incubated with biotinylated secondary antibody (1/1000), goat anti-rat (Vector Laboratories Inc., Burlingame, CA), and again washed with TBS-Tween. EBs were incubated with streptavidin-horseradish peroxidase (PerkinElmer Life Sciences), washed with TBS-Tween and H2O2, and the chromogen substance was added (AEC substrate kit, rat; Vector Laboratories Inc., Burlingame, CA). Alternatively, EBs were stained for CD41 (BD Biosciences), except that immunofluorescence (Alexa goat anti-rat 568; Molecular Probes) was adopted to visualize CD41-positive cells.

In Vitro Hematopoietic Differentiation—ES cells were differentiated according to a published procedure (30) using components from Stem Cell Technologies, Inc. (Vancouver, Canada). The procedure is depicted in supplemental Fig. 2. Two days prior to differentiation, ES cells were trypsinized and cultured in IMDM ES medium (Iscove’s modified Eagle’s medium with L-glutamine/HEPES (Invitrogen)), penicillin/streptomycin, 15% heat-inactivated fetal bovine serum (Invitrogen), 1× nonessential amino acids (Invitrogen), sodium pyruvate (Invitrogen), monothioglycerol (Sigma), and recombinant leu-
kemia inhibitory factor (Chemicon International, Temecula, CA). After 2 days in IMDM ES medium, the ES cells had reached 25–50% confluence and were then trypsinized and washed twice with IMDM 5% fetal calf serum. ES cells were then transferred to Petri dishes at a density of 1500 cells/ml in IMDM differentiation medium (Iscove's modified Eagle's medium/l-glutamine/HEPES (Invitrogen)), penicillin/streptomycin, 15% heat-inactivated fetal bovine serum (Invitrogen), monothioglycerol (Sigma), ascorbic acid (Sigma), monothioglycerol (Sigma), l-glutamine (Sigma), and protein-free hybridoma medium (Invitrogen) to form EBs. These were grown at 37 °C in 5% CO₂ for 7 days. Next, the media containing EBs were collected in Falcon tubes and the EBs allowed to sediment. Supernatants were removed, and EBs were trypsinized (3 min at 37 °C), after which the trypsin was inactivated by serum and EBs dispersed several times by an 18-gauge needle and 2–3 times through a 23-gauge needle. ES cells were counted and mixed by vortexing with methylcellulose medium (MethoCult/H23041, StemCell Technologies Inc., Vancouver, Canada) and plated with a syringe at a concentration of 10⁴ cells/ml. The MethoCult® M3434 medium contains Iscove’s modified Dulbecco’s medium with methylcellulose for solid support, fetal bovine serum, bovine serum albumin, recombinant human insulin, human transferrin, 2-mercaptoethanol, l-glutamine, recombinant mouse stem cell factor, recombinant mouse interleukin-3, recombinant mouse interleukin-6, and recombinant human erythropoietin to support hematopoietic colony growth. Cell colonies were scored after an additional 7–10 days of culture, thus giving a total differentiation time of 14–17 days.

**Statistical Analysis**—For real-time RT-PCR, β-actin-normalized cycle threshold values (as calculated by the Roche LightCycler version 2.0) were used to determine the relative expression levels of SHB.
the correct insertion of the neomycin gene, i.e. in the second allele of the first exon. Another two clones contained insertion in the first allele, whereas the second allele was untouched. For \( SHB^{+/−} \) cells, two sister clones with random insertion of the targeting construct were chosen. The mutations resulted in loss of SHB protein expression (Fig. 1C).

**Expression of Mesodermal Markers in \( SHB^{−/−} \) EBs**—Previous studies have suggested an involvement of SHB in vascular formation (27). We have presently analyzed the expression of two markers for early mesoderm at appropriate stages of spontaneous EB differentiation. The markers studied were Brachyury and Mix1. Brachyury and Mix1 are expressed in mesoderm, mesendoderm, and to a lesser extent in endoderm (31). Brachyury gene expression was rapidly down-regulated in the control EBs, whereas the \( SHB^{−/−} \) clone exhibited a significant delay in this response (Fig. 2). A similar pattern of delayed down-regulation of Mix1 gene expression was observed in the SHB-deficient clone, although the effect failed to reach statistical significance. The expression of later mesodermal markers relatively specific for the hematopoietic, vascular, and cardiac lineages was also studied. These were the hematopoietic markers Tal1 (32), CD41 (33, 34), and CD45 (35), the vascular markers CD31 (36, 37) and VEGFR-2 (7, 38), and the cardiac marker cardiac-actin (39). In the control EBs, all of these, except CD31, showed increased expression with time, which in the cases of Tal1, CD41, and cardiac-actin, peaked on day 8 (Fig. 2). The \( SHB^{−/−} \) clone showed reduced expression of most of these markers on day 6 or 8 of differentiation. On day 12 of differentiation, expression of Tal1 and CD41 peaked in the \( SHB^{−/−} \) clone and was higher than that of the control (Fig. 2). When comparing the \( SHB^{+/−} \) clone with the control and the \( SHB^{−/−} \) clones on day 12 of differentiation, it was observed that the loss of one \( SHB \) allele resulted in a response that was very similar to that of the complete \( SHB \) knock-out (Fig. 3). Particularly was expression of VEGFR-2, which has been shown to be essential for hematopoietic and vascular development (7), reduced in both the homozygous and heterozygous \( SHB \) knock-out ES cells (Fig. 3) at this time point. In addition, FGFR-1 expression was determined. SHB interacts with FGFR-1, which is a receptor that also plays a role for vascular and hematopoietic...
SHB and Embryonic Stem Cell Differentiation to Mesoderm

Our current intention was to study the potential of SHB^{-/-} ES cells to form blood vessels, because SHB previously has been shown to influence EB blood vessel formation. SHB^{-/-} or SHB^{+/+} EBs did not exhibit any significant morphological changes in their vasculatures compared with those of the SHB^{+/+} EBs when cultured under basal conditions (Fig. 5A). These EBs formed a vasculature that was primarily concentrated to the central core of the EB with sparse vascular structures extending toward the periphery. However, after culture in the presence of VEGF, the SHB^{-/-} and SHB^{+/+} EBs showed less developed peripheral plexa than the SHB^{+/+} EBs with fewer capillary structures (Fig. 5B). Similar results were obtained with the other SHB^{+/+} clone (data not shown). This finding is in the line with the previous study suggesting a role of SHB in regulating the development of vasculature in differentiating EBs (27).

---

**FIGURE 3.** Expression of hematopoietic and vascular markers in SHB-deficient EBs after 12 days of spontaneous differentiation. Graphs show the relative abundance of mRNA for each clone. Relative abundance of mRNA was calculated by formula: 100 x 2^{- ΔCT} for each clone, or 100 x 2^{- ΔΔCT} for each clone. Cytometry was normalized against β-actin. A, relative abundance of VEGFR-2 in SHB-deficient EBs (means ± S.E.; n = 6–7; ** indicates p < 0.01). B, FGFR-1 (means ± S.E.; n = 3–4; ** indicates p < 0.01). C, CD41 (means ± S.E.; n = 6; * indicates p < 0.05). D, Tal-1 (means ± S.E.; n = 6–8).

---

PCR data for the hematopoietic precursor markers CD41 and Tal-1 suggest a delayed up-regulation of gene expression but also an increased potential of SHB^{-/-} ES cells to differentiate into blood cells as their expression was elevated on day 12 of differentiation. To test whether SHB is important for in vitro erythropoiesis, EBs from the SHB^{-/-}, SHB^{+/+}, and SHB^{+/+} clones were analyzed for their capacity to form definitive blood cell colonies. Cell colonies were scored after days 7–11 of differentiation in methylcellulose preceded by a 7-day EB differentiation period allowing a total of 14–17 days of differentiation. SHB^{+/-} EBs generated fewer blood cell colonies than wild-type EBs. In three of four experiments, the SHB^{-/-} EBs also formed fewer blood cell colonies than the SHB^{+/+} clone (Fig. 6). This suggests that the main effect of SHB knock-out is delayed expression and decreased differentiation to the hematopoietic lineage.

**DISCUSSION**

In the present study, we have investigated the potential of SHB^{-/-} ES cells to differentiate in vitro. ES cells commonly differentiate in vitro in three-dimensional structures known as EBs, which similar to the embryo, contain cells from all three embryonal layers, i.e. ectoderm, endoderm, and mesoderm, but lack the complexity typical for the normal development of the organism. Murine ES cells can complement but not replace developmental studies on murine embryos.

Inactivation of the first exon in the SHB gene causes loss of SHB protein expression. The homologous recombination was not very efficient in the SHB locus. The low yield in obtaining a
clone after the second transfection could be due to the possibility that one allele is more permissive to the genetic replacement than the other one. Another explanation is that total SHB deficiency can cause less cell proliferation, and thus some SHB−/− clones will not expand sufficiently to be identified.

There is a close relationship between endoderm and mesoderm during development. Both of these germ layers originate in the primitive streak in a tissue referred to as mesendoderm (1). Also later during development, mesoderm and endoderm demonstrate interactions necessary for organ differentiation (4, 41). Recently, the existence of mesendoderm was described also in vitro (3). Our combined data support the role of SHB in mesodermal differentiation. Down-regulation of the early mesodermal marker Brachyury was paralleled by a delayed increase in the expression of several later mesodermal markers, such as VEGFR-2, Tal1, CD41, and cardiac-actin. Furthermore, expression of CD45, which is a hematopoietic marker expressed subsequently to CD41, was not increased in the SHB knock-out cells. The markers represent three different mesodermal lineages, and thus the data would suggest a generalized defect in mesodermal differentiation beyond early Brachyury expression.

Expression of VEGFR-2 was significantly decreased in both SHB+/− and SHB−/− EBs. VEGFR-2 is known as a factor involved in hematopoiesis and vasculogenesis in vivo and in vitro (29, 38, 42). When examining the SHB+/− and SHB−/− EBs for their ability to form blood vessels, it was noted that both clones were able to form blood vessels under basal conditions. These results are in contradiction with our previous study using the SH2 domain inactive mutant of SHB, R522K-SHB (27). R522K-SHB ES cells failed to form blood vessels regardless of what growth factor was added. The more prominent phenotype of the R522K-SHB ES cells may have several explanations. First, R522-SHB competes not only with the SHB protein but also

FIGURE 4. In situ detection of CD41+ by immunofluorescence after 4 + 7 days of differentiation. EBs were formed during preculture for 4 days and then cultured in chamber slides for 7 days before staining with anti-CD41. Original magnification in the left panels was 100×. Boxes indicate enlarged areas shown in the corresponding right panels.

FIGURE 5. In vitro blood vessel formation after 4 + 7 days of differentiation visualized by immunostaining with CD31 (red). A, blood vessel growth from EB core under basal condition. Magnification 20×. B, VEGF induces the formation of peripheral vascular plexa around EBs. Plexa are indicated by arrowheads, single vascular structures are indicated by arrows oriented toward the core of the EB. EB quadrants are shown; magnification 40×.
with other members of this protein family, such as SHD, SHE, SHF, and SHG. Second, in the SHB cells, these other members may compensate for certain SHB functions. Third, R522K-SHB may interfere with another pathway in which the wild-type SHB protein is not involved.

Both SHB+/− and SHB−/− EBs form less developed vascular plexa after VEGF stimulation. SHB is involved in the differentiation of endothelial Immortomouse brain endothelial cells (22) and in VEGF-induced migration of porcine aortic endothelial cells by binding to phosphorylated tyrosine 1175 (1173 in mouse) in the VEGFR-2 (19). The data thus suggest impaired signaling downstream of VEGFR-2 in SHB-deficient cells during blood vessel formation.

CD41 is known as a marker for hematopoietic progenitors (34, 43). The present finding that SHB-deficient EBs showed a delayed increase in expression that nevertheless reached higher levels of expression compared with control at day 12 could suggest an enhanced potential to differentiate to blood cells. This notion was not confirmed when performing a colony formation assay. Thus, the dominating feature is the delayed increase in CD41 expression that prevents further differentiation.

Increased CD41 expression without concomitant differentiation has previously been observed and was explained by the observed existence of two populations of CD41-positive cells, i.e. CD41+ “bright” and CD41+ “dim” (44). Thus, CD41+ dim cells were shown to have the capacity to differentiate into hematopoietic progenitors contrary to CD41+ bright cells, which showed a low capacity to differentiate. It is possible that the CD41-positive cells in the SHB EBs resemble primarily the CD41+ bright population of cells that fails to produce significant amounts of hematopoietic progenitors. Interestingly, the erythrocyte marker β-globin major, was decreased in R522K-SHB EBs.4

FGF-2 promotes the formation of slender blood vessels extending from the center to periphery in cultured EBs (29). Nevertheless, FGFR-1 knock-out EBs also produce vascular structures (40). We have not observed any morphological changes in SHB−/− EBs after the addition of FGF-2 (data not shown). The fact that both FGF-2 addition and FGFR-1 knock-out increase vessel formation in EBs could explain the lack of effect of SHB−/− in this context, especially because SHB may mediate certain aspects of FGFR-1 signaling (20).

In conclusion, the present data suggest that the SHB adapter protein is important for the formation of vascular structures and hematopoietic differentiation. This regulatory function operates at a stage later than initial mesodermal specification. Modulating SHB activity could serve a role in altering the developmental potential of differentiating ES cells.

Acknowledgments—We are grateful to the Uppsala University Transgene facility and Anne-Marie Olofsson for transfecting and expanding the ES cell clones. The loxP-plasmid backbone was kindly provided by Dr. Andy McMahon, Harvard University. We also appreciate comments by Dr. Leif Carlsson, Umeå University.

REFERENCES
1. Kimelman, D., and Griffin, K. J. (2000) Curr. Opin. Genet. Dev. 10, 350–356
2. Vallier, L., Reynolds, D., and Pedersen, R. A. (2004) Dev. Biol. 275, 403–421
4 V. Kriz, N. Ågren, C. K. Lindholm, S. Lenell, J. Saldeen, J. Mares, and M. Welsh, unpublished data.
3. Tada, S., Era, T., Furusawa, C., Sakurai, H., Nishikawa, S., Kinoshita, M., Nakao, K., and Chiba, T. (2005) Development (Camb.) 132, 4363–4374
4. Halonen, M., Kaestner, K. H., Martin-Parras, L., Sasaki, H., Betz, U. A., and Ang, S. L. (2002) Dev. Biol. 243, 20–33
5. Bielinska, M., Narita, N., Heikinheimo, M., Porter, S. B., and Wilson, D. B. (1996) Blood 88, 3720–3730
6. Palis, J., McGrath, K. E., and Kingsley, P. D. (1995) Blood 86, 156–163
7. Shalaby, F., Rossant, J., Yamaguchi, T. P., Gertsenstein, M., Wu, X. F., Breitman, M. L., and Schuh, A. C. (1995) Nature 376, 62–66
8. Kennedy, M., Firpo, M., Choi, K., Wall, C., Robertson, S., Kabrun, N., and Keller, G. (1997) Nature 386, 488–493
9. Choi, K., Kennedy, M., Kazarov, A., Papadimitriou, J. C., and Keller, G. (1998) Development (Camb.) 125, 725–732
10. Soria, B., Skoudy, A., and Martin, F. (2001) Diabetologia 44, 407–415
11. Fraichard, A., Chassande, O., Bilbaut, G., Dehay, C., Savatier, P., and Samarut, J. (1995) J. Cell Sci. 108, 3181–3188
12. Bagutti, C., Wobus, A. M., Fassler, R., and Watt, F. M. (1996) Dev. Biol. 179, 184–196
13. Abe, K., Nawa, H., Iwase, K., Takiguchi, M., Mori, M., Abe, S. I., and Yamamura, K. I. (1996) Exp. Cell Res. 229, 27–34
14. Welsh, M., Mares, J., Karlsson, T., Lavergne, C., Breant, B., and Claesson-Welsh, L. (1994) Oncogene 9, 19–27
15. Karlsson, T., Songyang, Z., Landgren, E., Lavergne, C., Di Fiore, P. P., Anafi, M., Pawson, T., Cantley, L. C., Claesson-Welsh, L., and Welsh, M. (1995) Oncogene 10, 1475–1483
16. Welsh, M., Songyang, Z., Frantz, J. D., Trub, T., Reedquist, K. A., Karlsson, T., Miyazaki, M., Cantley, L. C., Band, H., and Shoelson, S. E. (1998) Oncogene 16, 891–901
17. Lindholm, C. K., Henriksson, M. L., Hallberg, B., and Welsh, M. (2002) Eur. J. Biochem. 296, 3279–3288
18. Lu, L., Anneren, C., Reedquist, K. A., Bos, I. L., and Welsh, M. (2000) Exp. Cell Res. 259, 370–377
19. Holmqvist, K., Cross, M. J., Rolny, C., Hagerkvist, R., Rahimi, N., Matsumoto, T., Claesson-Welsh, L., and Welsh, M. (2004) J. Biol. Chem. 279, 22267–22275
20. Cross, M. J., Lu, L., Magnusson, P., Nyqvist, D., Holmqvist, K., Welsh, M., and Claesson-Welsh, L. (2002) Mol. Biol. Cell 13, 2881–2893
21. Lindholm, C. K., Gyle, E., Zhang, W., Samelson, L. E., and Welsh, M. (1999) J. Biol. Chem. 274, 28050–28057
22. Lu, L., Holmqvist, K., Cross, M., and Welsh, M. (2002) Cell Growth & Differ. 13, 141–148
23. Welsh, M., Christmasson, L., Karlsson, T., Sandler, S., and Welsh, N. (1999) Mol. Med. 5, 169–180
24. Karlsson, T., and Welsh, M. (1996) Oncogene 13, 955–961
25. Dixielius, J., Larsson, H., Sasaki, T., Holmqvist, K., Lu, L., Engstrom, A., Triml, R., Welsh, M., and Claesson-Welsh, L. (2000) Blood 95, 3403–3411
26. Koz, V., Anneren, C., Lai, C., Karlsson, J., Mares, J., and Welsh, M. (2003) Exp. Cell Res. 286, 40–56
27. Rolny, C., Lu, L., Agren, N., Nilsson, I., Roe, C., Webb, G. C., and Welsh, M. (2005) Exp. Cell Res. 308, 381–393
28. Wobus, A. M., Maomi, G., Shan, J., Wellner, M., Rohlwedel, J., Li, G., Fleischmann, B., Katus, H. A., Hescheler, J., and Franzen, W. M. (1997) J. Mol. Cell. Cardiol. 29, 1525–1539
29. Magnusson, P., Rolny, C., Jakobsson, L., Wikner, C., Wu, Y., Hicklin, D. J., and Claesson-Welsh, L. (2004) J. Cell Sci. 117, 1513–1523
30. Carlsson, L., Wanzioh, E., Pinto do, O. P., and Kolterud, A. (2003) Methods Enzymol. 365, 202–214
31. Yasunaga, M., Tada, S., Torikai-Nishikawa, S., Nakano, Y., Okada, M., Jakt, L. M., Nishikawa, S., Chiba, T., and Era, T. (2005) Nat. Biotechnol. 23, 1542–1550
32. Callianpur, A. R., Jordan, J. E., and Brandt, S. J. (1994) Blood 83, 1200–1208
33. Ferkowicz, M. J., Starr, M., Xie, X., Li, W., Johnson, S. A., Shelley, W. C., Morrison, P. R., and Yoder, M. C. (2003) Development (Camb.) 130, 4393–4403
34. Mitjavila-Garcia, M. T., Cailleret, M., Godin, I., Nogueira, M. M., Cohen-Solal, K., Schiavon, V., Lechuse, Y., Le Pester, F., Lagrue, A. H., and Vainchenker, W. (2002) Development (Camb.) 129, 2003–2013
35. Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Tims, E., Pfeffer, K., Ohashi, P. S., Thomas, M. L., Furlonger, C., Paige, C. J., and Mak, T. W. (1993) Cell 74, 143–156
36. Drake, C. J., and Fleming, P. A. (2000) Blood 95, 1671–1679
37. Baldwin, H. S., Shen, H. M., Yan, H. C., DeLisser, H. M., Chung, A., Mickanin, C., Trask, T., Kirschbaum, N. E., Newman, P. J., Albelda, S. M., and Buck, C. A. (1994) Development (Camb.) 120, 2539–2553
38. Shalaby, F., Ho, I., Stanford, W. L., Fischer, K. D., Schuh, A. C., Schwartz, L., Bernstein, A., and Rossant, J. (1997) Cell 89, 981–990
39. Schuldiner, M., Yanuka, O., Itskovitz-Eldor, J., Melton, D. A., and Benvenisty, N. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 11307–11312
40. Magnusson, P. U., Ronca, R., Dell’Era, P., Carlstedt, P., Jakobsson, L., Partanen, J., Dimberg, A., and Claesson-Welsh, L. (2005) Arterioscler. Thromb. Vasc. Biol. 25, 944–949
41. Kumar, M., Jordan, N., Melton, D., and Grapin-Botton, A. (2003) Dev. Biol. 259, 109–122
42. Kabrun, N., Buhring, H. J., Choi, K., Ullrich, A., Risau, W., and Keller, G. (1997) Development (Camb.) 124, 2039–2048
43. Emambokus, N. R., and Frampton, J. (2003) Immunity 19, 33–45
44. Otani, T., Inoue, T., Tsuji-Takayama, K., Ijiri, Y., Nakamura, S., Motoda, R., and Orita, K. (2005) Exp. Hematol. 33, 632–640