A

B

FIGURE 6. **AML1 forms different transcriptional complexes on the c-mpl promoter in hematopoietic stem/progenitor cells and megakaryocytes.** *A,* experimental design of ChIP-ReChIP assays. Murine Lin⁻Sca1⁺ cells were cultured for 3 days with mSCF (50 ng/ml), mIL-3 (10 ng/ml), and hTPO (50 ng/ml). These cultured cells and CMK cells were cross-linked and subjected to the ChIP-ReChIP assays. *B,* after the second immunoprecipitation (*IP*), PCR analyses were performed using the primer set shown in Fig. 2 with the eluted DNA as a template.

transcriptional complex with various molecules, we hypothesized that AML1 may change the binding partner included in the transcriptional complex, thereby regulating the c-*mpl* promoter either positively or negatively according to cell types. To assess this hypothesis, we performed ChIP-ReChIP assays. Nuclear extracts were prepared from 3-day-cultured Lin⁻Sca1⁺ cells and CMK cells. After the first ChIP with the AML1 Ab, the eluted samples, including the transcriptional complex of AML1, were re-immunoprecipitated by the anti-p300 Ab and anti-mSin3A Ab respectively (Fig. 6*A*). As shown in Fig. 6*B, upper panel,* the anti-mSin3A Ab but not the anti-p300 Ab immunoprecipitated the c-*mpl* promoter from the transcriptional complex of AML1 obtained from Lin⁻Sca1⁺ cells. In contrast, the c-*mpl* promoter was immunoprecipitated by the anti-p300 Ab but not by the anti-mSin3A Ab from the transcriptional complex of AML1 isolated from CMK cells (Fig. 6*B*). These results suggest that AML1 represses the c-*mpl* promoter by forming a complex with a transcriptional corepressor mSin3A in hematopoietic stem/progenitor cells, although it activates the c-*mpl* promoter by forming a complex with a transcriptional activator p300 in megakaryocytic CMK cells.

AML1dC Enhances TPO Signaling and TPO-dependent Colony Forming Activity—To assess the biologic significance of the AML1dC-enhanced c-Mpl expression in hematopoietic stem/progenitor cells, we initially compared TPO-induced tyrosine phosphorylation of STAT5 between AML1dC- and Mock-transduced KSL cells by flow cytometry. As a result, we found that the stimulation with TPO for 15 min activated STAT5

more effectively in AML1dC-transduced cells than in Mock-transduced cells (% of activated cells: AML1dC 51.8% *versus* Mock 32.2%) (Fig. 7*A*). Meanwhile, TPO-induced STAT5 activation in AML1b-transduced cells was distinctly attenuated compared with Mock-transduced cells (% of activated cells, AML1b 11.1% *versus* Mock 32.2%). Also, we performed colony assays using these cells under several conditions with or without TPO. As shown in Fig. 7*B*, although AML1dC- and Mock-transduced KSL cells developed almost equivalent numbers of hematopoietic colonies in the absence of TPO, AML1dC-transduced KSL cells yielded more and larger colonies than Mock-transduced KSL cells in the presence of TPO. In particular, CFU-GEMM was formed from AML1dC-transduced KSL cells but not from Mock-transduced KSL cells. These results suggest that the augmented c-Mpl expression by AML1dC led to the enhanced proliferation (in part, self-renewal) and survival of KSLs through the TPO/c-Mpl signaling (Fig. 7*B*).

DISCUSSION

Because both AML1 and TPO/c-Mpl signaling play important roles in the growth of HSCs and megakaryopoiesis, we assumed that AML1 might regulate TPO/c-Mpl signaling. Also, in a recent paper, Heller *et al.* (31) reported that platelet surface c-Mpl expression was decreased in FPD/AML patients, suggesting that AML1 would augment c-Mpl expression in megakaryocytes. To clarify this relationship, in this study we performed luciferase assays, EMSA, and ChIP assays with the c-*mpl* promoter. As a result, we found that AML1 directly binds to the proximal AML-binding sequence between −137 and −122 bp of the c-*mpl* promoter, thereby regulating its activity. In agreement with the suggestive data by Heller *et al.* (31), AML1 activated the c-*mpl* promoter in luciferase assays using 293T cells and HeLa cells. However, the enforced expression of a dominant-negative form of AML1, AML1dC, in KSL cells by the retrovirus system enhanced c-Mpl expression in hematopoietic stem/progenitor cells and exogenous AML1b transduction into KSL cells and attenuated c-Mpl expression and TPO-induced STAT5 activation. Also, the induced expression of AML1dC during the development of hematopoietic cells in the OP9 system enhanced c-Mpl expression on hematopoietic stem/progenitor cells. Furthermore, early hematopoietic cells that derived from AML1⁺/⁻ ES cells expressed c-Mpl more intensively than those that developed from WT ES cells. These results suggest that AML1 is a negative regulator of c-Mpl expression in these cells, which is opposite to its role in megakaryocytes. As for this inconsistent result observed in hematopoietic stem/progenitor cells, we speculate that AML1 would be able to regulate the c-*mpl* promoter both positively and negatively according to cell types. In fact, AML1 and its heterodimeric partner, PEBP2β, have been reported to form a

FIGURE 5. **Effects of AML1dC on c-Mpl expression on hematopoietic progenitor cells and megakaryocytes and megakaryopoiesis.** *A,* experimental design using the OP9 system. ES cells were deprived of leukemia inhibitory factor and cultured on OP9 cells for 4.5 days. Then Flk-1⁺ cell we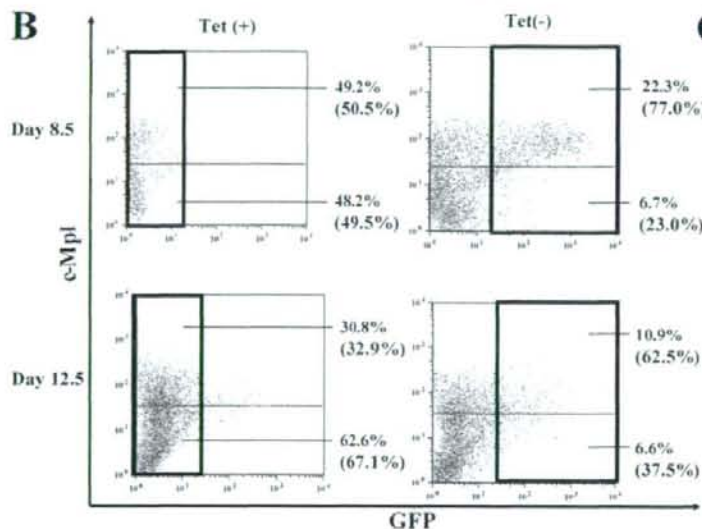re sorted, replated onto OP9 cells, and cultured with mSCF and hTPO (for the analysis of c-Mpl expression in hematopoietic stem/progenitor cells) or only hTPO (for the analysis of meg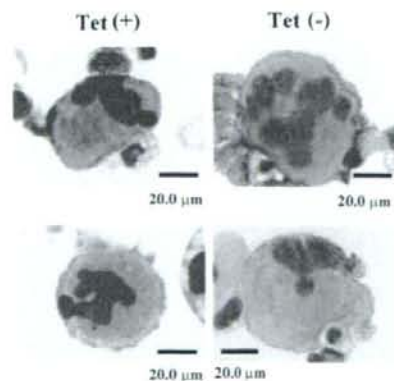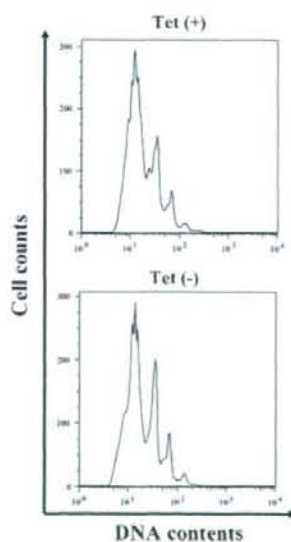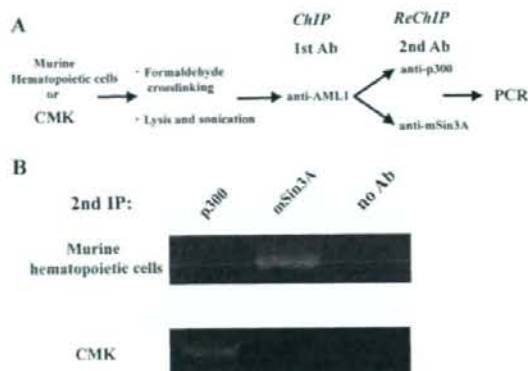akaryocytic differentiation) with or without Tet for the time indicated. *B,* c-Mpl expression of nonadherent cells was examined by the direct immunofluorescence method on day 8.5 and day 12.5. The percentage of each fraction is indicated. The relative frequency of GFP⁻ fraction in cultured cells with Tet and the relative frequency of GFP⁺ fraction in cultured cells without Tet were shown in *parentheses. C,* c-Mpl expression on early hematopoietic cells that derived from WT and AML1⁺/⁻ ES cells on day 8.5. *D* and *E,* after 12.5-day cultures with TPO, megakaryocytic cells, which derived from ES cells expressing AMLdC, were subjected to morphological analysis (*D*), and DNA content analysis by propidium iodide staining (*E*).

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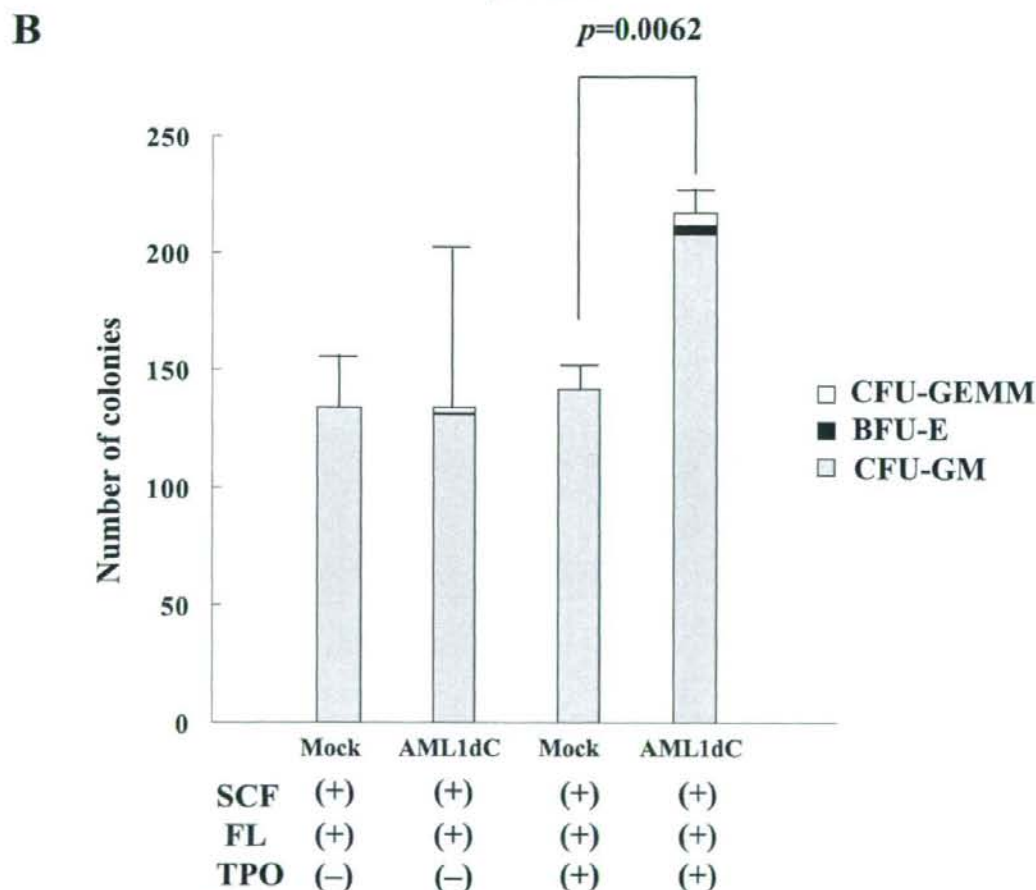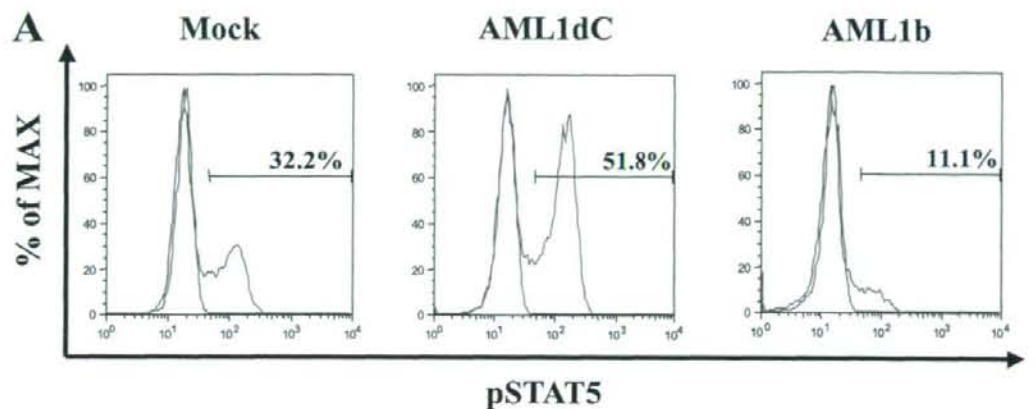
FIGURE 7. **AML1dC enhances TPO signaling and TPO-dependent colony forming activity.** *A*, after 15-min of TPO stimulation, tyrosine phosphorylation of STAT5 was examined by FACS in Mock-, AML1dC-, and AML1b-transduced cells, which are gated as a GFP+ fraction. *Red line*, with TPO stimulation. *Blue line*, without TPO stimulation. *B*, 2 days after retrovirus infection, GFP+ cells (1000 cells/dish) were seeded into the methylcellulose media with indicated cytokines. The number of CFU-GM was counted on day 7 and those of BFU-E and CFU-GEMM on day 12. The results are shown as mean ± S.D. of triplicate experiments. *CFU-GEMM (open square); BFU-E (closed square); CFU-GM (gray square).*

transcriptional complex with various molecules and to change its function dependently on cell types. When AML1 forms a complex with p300/CBP and MOZ, this complex strongly stimulates AML1-mediated transcription (48). On the other hand, when AML1 combines with mSin3A, this complex works as a transcriptional repressor (49). In agreement with this speculation, we found that AML1 forms a complex with mSin3A on the c-*mpl* promoter in hematopoietic stem/progenitor cells, whereas it formed a complex with p300 on the same promoter in megakaryocytic cells (Fig. 6*B*). These results suggest that AML1 plays distinct roles in the regulation of the c-*mpl* promoter dependent on cell types by changing the binding partner.

In a previous paper, Ichikawa *et al.* (17) reported that conditional targeting of AML1 in adult mice led to the impaired polyploidization of megakaryocytes, resulting in the low platelet production. Also, patients with hereditary FPD/AML, which was caused by the heterozygous point mutations of the *AML1* gene or the *PEBP2β* gene revealed low platelet numbers in the peripheral blood (19). To clarify the roles of AML1 in megakaryocytic maturation, several studies have been performed. Consequently, Bernardin-Fried *et al.* (50) found that AML1 directly activates the cyclin D3 promoter, thereby enhancing DNA synthesis required for polyploidization. Also, Goldfarb and co-workers (51, 52) showed that AML1 binds to and activates the promoter of megakaryocyte-specific genes, αIIb integrin, and glycoprotein Ibα, in combination with a transcription factor specific for the erythroid/megakaryocyte lineage, GATA-1, thereby promoting phenotypic maturation of megakaryocytes (51, 52). Most AML1 mutations observed in FPD/AML and AML are clustered within the Runt domain in the N-terminal region (19, 53–55). Heterozygous Runt domain mutations show haploinsufficient phenotype because of their reduced DNA binding and PEBP2β binding (30, 53). On the other hand, because C-terminal deletion mutants of AML1 have enhanced DNA-binding potential, they strongly suppress wild-type AML1 function through the blocking of its DNA binding in a dominant-negative manner (30). In line with this result, we also found that AMLdC lacking the C-terminal amino acids 224–453 dominant-negatively suppressed DNA binding of WT AML1 in EMSA using nuclear extracts of 293T cells. However, in this study, we found that AML1dC scarcely influenced the morphology or polyploidization of megakaryocytes. This result seems to be at variance with previous reports indicating the importance of AML1 in megakaryopoiesis (as described above). However, because an apparent abnormality was not detected in megakaryopoiesis and platelet production in AML1 heterozygous knock-out mice (14), it was speculated that pure haploinsufficiency of AML1 would not impair maturation of megakaryocytes or platelet production. Although genuine haploinsufficiency of AML1 was observed in some cases, it was also speculated that a greater majority of mutant AML1 proteins are assumed to act in a dominant-negative manner (56). So, at present, it remains unknown to what degree AML1 activity must be suppressed to cause the defect in megakaryopoiesis. In addition, there is a possibility that although AML1dC was found to act as a dominant-negative suppressor of AML1 in 293T cells in this study, AML1dC protein might be far more labile than wild-type AML1 in megakaryocytes. Alter-

natively, it is also possible that AMLdC might reveal some unknown biologic effect on megakaryopoiesis in combination with other transcriptional regulators such as GATA-1. Although the precise mechanism remains to be clarified, our data suggest that AMLdC lacking the C-terminal amino acids 224–453 by itself would not be responsible for the impaired megakaryopoiesis in the original MDS patient. Further studies using several C-terminal mutants are required to clarify the roles of mutants of AML1 in impaired megakaryopoiesis in MDS patients.

Conditional deletion of AML1 in adult mice leads to the expansion of the HSC compartment and reduction of common lymphoid progenitors (CLPs) as well as impaired megakaryopoiesis (17, 18), suggesting that AML1 enhances differentiation of HSC toward the CLP compartment. Meanwhile, c-*mpl* mRNA is expressed on HSCs and common myeloid progenitors but not on CLPs (16). However, considering our result that AML1-deficient hematopoietic stem/progenitor cells are hyper-responsive to TPO because of the enhanced c-Mpl expression, these reports may simply indicate that AML1-deficient HSCs and common myeloid progenitors would overgrow as compared with CLPs in response to the TPO stimulation. Also, it was speculated that MDS stem cells harboring AML1dC might be hyper-responsive to TPO, leading to the accumulation of oxidative stress that causes second genetic abnormalities.

In conclusion, we show here that AML1 acts as a negative regulator of c-Mpl expression in hematopoietic stem/progenitor cells which is opposite to its role in megakaryocytes. Also, we found that hematopoietic stem/progenitor cells harboring AML1dC were hyperproliferative in response to TPO. Further studies focusing on the roles of various types of AML1 mutants would be useful to clarify the physiologic roles for AML1 and to understand the pathophysiology of MDS.

REFERENCES

1. Miyoshi, H., Shimizu, H., Kozu, T., Maseki, N., Kaneko, Y., and Ohki, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 10431–10434
2. Golub, T. R., Barker, G. F., Bohlander, S. K., Hebert, S. W., Ward, D. C., Bray-Ward, P., Morgan, E., Raimondi, S. C., Rowley, J. D., and Gilliland, D. G. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 4917–4921
3. Osato, M. (2004) *Oncogene* **23**, 4284–4296
4. Sasaki, K., Yagi, H., Bronson, R. T., Tominaga, K., Matsunashi, T., Deguchi, K., Tani, Y., Kishimoto, T., and Komori, T. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 12359–12363
5. Wang, Q., Stacy, T., Miller, J. D., Lewis, A. F., Gu, T. L., Huang, X., Bushweller, J. H., Bories, J. C., Alt, F. W., Ryan G., Liu, P. P., Wynshaw-Boris, A., Binder, M., Marín-Padilla, M., Sharpe, A. H., and Speck, N. A. (1996) *Cell* **87**, 697–708
6. Niki, M., Okada, H., Takano, H., Kuno, J., Tani, K., Hibino, H., Asano, S., Ito, Y., Satake, M., and Noda, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 5697–5702
7. Cameron, S., Taylor, D. S., TePas, E. C., Speck, N. A., and Mathey-Prevot, B. (1994) *Blood* **83**, 2851–2859
8. Otto, F., Lübbert, M., and Stock, M. (2003) *J. Cell. Biochem.* **89**, 9–18
9. Taniuchi, I., Osato, M., Egawa, T., Sunshine, M. J., Bae, S. C., Komori, T., Ito, Y., and Littman, D. R. (2002) *Cell* **111**, 621–633

10. Woolf, E., Xiao, C., Fainaru, O., Lotem, J., Rosen, D., Negreanu, V., Bernstein, Y., Goldenberg, D., Brenner, O., Berke, G., Levanon, D., and Groner, Y. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 7731–7736

11. Takahashi, A., Satake, M., Yamaguchi-Iwai, Y., Bae, S. C., Lu, J., Maruyama, M., Zhang, Y. W., Oka, H., Arai, N., Arai, K., and Ito, Y. (1995) *Blood* **86**, 607–616

12. Zhang, D. E., Fujioka, K., Hetherington, C. J., Shapiro, L. H., Chen, H. M., Look, A. T., and Tenen, D. G. (1994) *Mol. Cell. Biol.* **14**, 8085–8095

13. Jiang, H., Zhang, F., Kurozu, T., and Peterlin, B. M. (2005) *Mol. Cell. Biol.* **25**, 10675–10683

14. Okuda, T., van Deursen, J., Hiebert, S. W., Grosveld, G., and Downing, J. R. (1996) *Cell* **84**, 321–330

15. North, T. E., de Bruijn, M. F., Stacy, T., Talebian, L., Lind, E., Robin, C., Binder, M., Dzierzak, E., and Speck, N. A. (2002) *Immunity* **16**, 661–672

16. Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000) *Nature* **404**, 193–197

17. Ichikawa, M., Asai, T., Chiba, S., Kurokawa, M., and Ogawa, S. (2004) *Nat. Med.* **10**, 299–304

18. Growney, J. D., Shigematsu, H., Li, Z., Lee, B. H., Adelsperger, J., Rowan, R., Curley, D. P., Kutok, J. L., Akashi, K., Williams, I. R., Speck, N. A., and Gilliland, D. G. (2005) *Blood* **106**, 494–504

19. Song, W. J., Sullivan, M. G., Legare, R. D., Hutchings, S., Tan, X., Kufrin, D., Ratajczak, J., Resende, I. C., Haworth, C., Hock, R., Loh, M., Felix, C., Roy, D. C., Busque, L., Kurnit, D., Willman, C., Gewirtz, A. M., Speck, N. A., Bushweller, J. H., Li, F. P., Gardiner, K., Poncz, M., Maris, J. M., and Gilliland D. G. (1999) *Nat. Genet.* **23**, 166–175

20. Hirai, H. (2003) *Jpn. J. Clin. Oncol.* **33**, 153–160

21. Paquette, R. L., Landaw, E. M., Pierre, R. V., Kahan, J., Lübbert, M., Lazcano, O., Isaac, G., McCormick, F., and Koeffler, H. P. (1993) *Blood* **82**, 590–599

22. Yokota, S., Kiyoi, H., Nakao, M., Iwai, T., Misawa, S., Okuda, T., Sonoda, Y., Abe, T., Kahsima, K., Matsuo, Y., and Naoe, T. (1997) *Leukemia (Basingstoke)* **11**, 1605–1609

23. Uchida, T., Kinoshita, T., Nagai, H., Nakahara, Y., Saito, H., Hotta, T., and Murate, T. (1997) *Blood* **90**, 1403–1409

24. Quesnel, B., Guillerm, G., Vereecque, R., Wattel, E., Preudhomme, C., Bauters, F., Vanrumbeke, M., and Fenaux, P. (1998) *Blood* **91**, 2985–2990

25. Sugimoto, K., Hirano, H., Toyoshima, H., Chiba, S., Mano, H., Takaku, F., Yazaki, Y., and Hirai, H (1993) *Blood* **81**, 3022–3026

26. Russell, M., List, A., Greenberg, P., Woodward, S., Glinsmann, B., Parganas, E., Ihle, J., and Taetle, R. (1994) *Blood* **84**, 1243–1248

27. Willman, C. L., Sever, C. E., Pallavicini, M. G., Harada, H., Tanaka, N., Slovak, M. L., Yamamoto, H., Harada, K., Meeker, T. C., List, A. F., *et al.* (1993) *Science* **259**, 968–971

28. Harada, H., Harada, Y., Tanaka, H., Kimura, A., and Inaba, T. (2003) *Blood* **101**, 673–680

29. Yoneda-Kato, N., Look, A. T., Kirstein, M. N., Valentine, M. B., Raimondi, S. C., Cohen, K. J., Carroll A. J., and Morris, S. W. (1996) *Oncogene* **12**, 265–275

30. Harada, H., Harada, Y., Niimi, H., Kyo, T., Kimura, A., and Inaba, T. (2004) *Blood* **103**, 2316–2324

31. Heller, P. G., Glembotsky, A. C., Gandhi, M. J., Cummings, C. L., Pirola, C. J., Marta, R. F., Kornblihtt, L. I., Drachman, J. G., and Molinas, F. C. (2005) *Blood* **105**, 4664–4670

32. Kaushansky, K., Lok, S., Holly, R. D., Broudy, V. C., Lin, N., Bailey, M. C., Forstrom, J. W., Buddle, M. M., Oort, P. J., Hagen F. S., Roth, G. J., Papay-

annopoulou, T., and Foster, D. C. (1994) *Nature* **369**, 568–571

33. Solar, G. P., Kerr, W. G., Zeigler, F. C., Hess, D., Donahue, C., de Sauvage, F. J., and Eaton, D. L. (1998) *Blood* **92**, 4–10

34. Fox, N., Priestley, G., Papayannopoulou, T., and Kaushansky, K (2002) *J. Clin. Investig.* **110**, 389–394

35. Shimizu, K., Kitabayashi, I., Kamada, N., Abe, T., Maseki, N., Suzukawa, K., and Ohki, M. (2000) *Blood* **96**, 288–296

36. Gu, T. L., Goetz, T. L., Graves, B. J., and Speck, N. A. (2000) *Mol. Cell. Biol.* **20**, 91–103

37. Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R. G., and Kanakura, Y. (1999) *EMBO J.* **18**, 1367–1377

38. Satoh, Y., Matsumura, I., Tanaka, H., Ezoe, S., Sugahara, H., Mizuki, M., Shibayama, H., Ishiko, E., Ishiko, J., Nakajima, K., and Kanakura, Y. (2004) *J. Biol. Chem.* **279**, 24986–24993

39. Meyers, S., Downing, J. R., and Hiebert, S. W. (1993) *Mol. Cell. Biol.* **13**, 6336–6345

40. Jalvy, S., Renault, M. A., Lam Shang Leen, L., Belloc, I., Reynaud, A., Gadeau, A. P., and Desgranges, C. (2007) *Circ. Res.* **100**, 1292–1299

41. Wei, X., Xu, H., and Kufe, D. (2007) *Cancer Res.* **67**, 1853–1858

42. Nakano, T., Kodama, H., and Honjo, T. (1994) *Science* **265**, 1098–1101

43. Niwa, H., Burdon, T., Chambers, I., and Smith, A. (1998) *Genes Dev.* **12**, 2048–2060

44. Era, T., and Witte, O. N. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1737–1742

45. Nakata, S., Matsumura, I., Tanaka, H., Ezoe, S., Satoh, Y., Ishikawa, J., Era, T., and Kanakura, Y. (2004) *J. Biol. Chem.* **279**, 55578–55586

46. Vangala, R. K., Heiss-Neumann, M. S., Rangatia, J. S., Singh, S. M., Schoch, C., Tenen, D. G., Hiddemann, W., and Behre, G. (2003) *Blood* **101**, 270–277

47. Okuda, T., Takeda, K., Fujita, Y., Nishimura, M., Yagyu, S., Yoshida, M., Akira, S., Downing, J. R., and Abe, T. (2000) *Mol. Cell. Biol.* **20**, 319–328

48. Kitabayashi, I., Aikawa, Y., Nguyen, L. A., Yokoyama, A., and Ohki, M. (2001) *EMBO J.* **20**, 7184–7196

49. Lutterback, B., Westendorf, J. J., Linggi, B., Isaac, S., Seto, E., and Hiebert, S. W. (2000) *J. Biol. Chem.* **275**, 651–656

50. Bernardin-Fried, F., Kummalue, T., Leijen, S., Collector, M. I., Ravid, K., and Friedman, A. D. (2004) *J. Biol. Chem.* **279**, 15678–15687

51. Elagib, K. E., and Goldfarb, A. N. (2007) *Crit. Rev. Eukaryotic Gene Expression* **17**, 271–280

52. Elagib, K. E., Racke, F. K., Mogass, M., Khetawat, R., Delehanty, L. L., and Goldfarb, A. N. (2003) *Blood* **101**, 4333–4341

53. Michaud, J., Wu, F., Osato, M., Cottles, G. M., Yanagida, M., Asou, N., Shigesada, K., Ito, Y., Benson, K. F., Raskind, W. H., Rossier, C., Antonarakis, S. E., Israels, S., McNicol, A., Weiss, H., Horwitz, M., and Scott, H. S. (2002) *Blood* **99**, 1364–1372

54. Osato, M., Asou, N., Abdalla, E., Hoshino, K., Yamasaki, H., Okubo, T., Suzushima, H., Takatsuki, K., Kanno, T., Shigesada, K., and Ito, Y. (1999) *Blood* **93**, 1817–1824

55. Preudhomme, C., Warot-Loze, D., Roumier, C., Grardel-Duflos, N., Garand, R., Lai, J. L., Dastugue, N., Macintyre, E., Denis, C., Bauters, F., Kerckaert, J. P., Cosson, A., and Fenaux, P. (2000) *Blood* **96**, 2862–2869

56. Osato, M., Yanagida, M., Shigesada, K., and Ito, Y. (2001) *Int. J. Hematol.* **74**, 245–251

57. Kimura, S., Roberts, A. W., Metcalf, D., and Alexander, W. S. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 1195–1200

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Soluble Frizzled-Related Protein 1 Is Estrogen Inducible in Bone Marrow Stromal Cells and Suppresses the Earliest Events in Lymphopoiesis[1]

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It has long been known that lymphopoiesis is transiently suppressed during pregnancy, which can be experimentally simulated by estrogen treatment. We now confirm with Rag1/GFP reporter mice that early lymphoid progenitors in the lineage marker⁻ c-kit^{high} $ScaI^+$, hematopoietic stem cell-enriched fraction of bone marrow are particularly depressed in these circumstances. Hematopoietic and environmental cells are both potential hormone targets and, because of this complexity, very little is known regarding mechanisms. We have now identified soluble Frizzled-related protein (sFRP)1 as an estrogen-inducible gene in stromal cells, whose expression corresponded to inability to support lymphopoiesis. Bone-lining stromal cells express sFRP1, and the transcripts were elevated by pregnancy or estrogen injection. Estrogen receptor-α was essential for both lymphoid suppression and induction of the sFRP family. SFRP1 has been mainly described as an antagonist for complex Wnt signals. However, we found that sFRP1, like Wnt3a, stabilized β-catenin and blocked early lymphoid progression. Myeloerythroid progenitors were less affected by sFRP1 in culture, which was similar to estrogen with respect to lineage specificity. Hematopoietic stem cells expressed various Frizzled receptors, which markedly declined as they differentiated to lymphoid lineage. Thus, hormonal control of early lymphopoiesis in adults might partly relate to sFRP1 levels. *The Journal of Immunology*, 2008, 181: 6061–6072.

L ymphocytes are produced throughout life from self-renewing hematopoietic stem cells (HSC)[3] and are indispensable elements of the immune system. Much has been learned about molecular mechanisms that regulate lymphocyte differentiation (1, 2). For example, it is now clear that lymphoid gene expression in progenitor cells is determined by levels and combinations of key transcription factors cooperating or cross-competing with each other in a hierarchy (3). HSC give rise to multipotent progenitors (MPP) that lack extensive self-renewal ability and eventually segregate into the various blood cell lineages. Lymphoid-primed MPP in the mouse represent a rare lineage marker-negative (Lin⁻) $ScaI^+$ c-kit^{high} CD150⁻ CD27⁺ Flk-2⁺ subset of bone marrow (BM) cells, and some fraction of them replenish all of the lymphocyte populations, including B cells (4). Knowledge about extracellular cues that direct early steps in lymphocyte formation is incomplete, but cytokines such as stem cell factor and Flt-3 ligand are certainly important. Cell adhesion molecules allow stem and progenitor cells to be in proximity to stromal cells that produce those factors, as well as ligands in the extremely complex Notch and Wnt families that control many differentiation events (5).

There is evidence that sex steroids play important regulatory roles in lymphocyte production. All cells in the lymphoid differentiation series decline dramatically in the BM of pregnant mice, whereas they are abnormally elevated in castrated male, ovariectomized female, hypogonadal, and androgen receptor-deficient animals (6). Treatment with estrogen does not precisely simulate all of the pregnancy-related changes, but it restores B lymphopoiesis in hypogonadal mice to the normal range and selectively depletes lymphoid progenitors in normal animals (7, 8). In fact, sensitivity to estrogen made it possible to identify primitive cells in the MPP fraction that can generate T and B lymphocytes (9). These early lymphoid progenitors (ELP) are not homogenous, but express different combinations of lymphoid genes, such as TdT and Rag1 (10).

BM contains multiple cell types that could be hormone targets, accounting in part for the modest progress made in determining exactly how sex steroids regulate lymphopoiesis. Lymphoid progenitors express estrogen and androgen receptors in a developmental age-dependent manner (11). Furthermore, they are directly sensitive to estrogen in stromal cell-free, serum-free cultures (12). Transplantation studies with estrogen receptor (ER)-deficient mice also implicated hematopoietic cells in this process (13). However,

other findings suggest that stromal cells can make suppressive factors for lymphopoiesis when exposed to hormone (14). We have now identified one such substance as soluble Frizzled-related protein (sFRP)1.

sFRPs are among several types of extracellular regulators for Wnt signaling (15). The five members of this family contain a characteristic cysteine-rich domain (CRD) that shares 30–50% homology with Frizzled for Wnt. Several studies have shown that sFRPs can antagonize Wnt signaling by interrupting the binding of Wnt to Frizzled (16, 17). However, it is not clear whether all sFRPs down-regulate Wnt signaling or how specific these interactions are. There are at least 19 Wnt proteins that bind to 9 Frizzled or other receptors and exhibit their activities depending on receptor context (18). Furthermore, recent studies demonstrated that sFRPs can even function through Wnt-independent mechanisms (19, 20). These complex observations make it extremely difficult to define the physiological role of sFRPs. They are known to be expressed in BM (21), and may have a role in maintenance of bone density, but little is known about their importance for normal blood cell formation.

At least one Wnt ligand, Wnt3a, can slow the differentiation of HSC, and purified HSC from Bcl-2 transgenic mice were propagated in culture with this as the primary stimulus (22). The best studied of several Wnt signaling pathways involves stabilization of intracellular β-catenin that moves to the nucleus and activates genes through association with T cell-specific factor/lymphoid-enhancer factor transcription factors (23). Experimental introduction of constitutively active β-catenin preserves the multipotential status of HSC and restores primitive properties to committed myeloid and lymphoid progenitors (24, 25). Wnt5a appears to limit late stages of B lymphopoiesis and may function as a tumor suppressor (26). In contrast, stromal cell production of Wnt5a promotes the formation of primitive lymphoid cells (27). It is interesting that Wnt5a can inhibit Wnt3a function in a β-catenin signaling pathway-independent fashion (28). Alternatively, and depending on which Frizzled receptor is expressed, the same ligand can stimulate canonical β-catenin pathway (29). These examples account for the interest in Wnt family molecules as potential regulators of lymphohematopoiesis. However, they also illustrate the complexity that accounts for the difficulty in ascribing roles under normal steady-state conditions.

An initial goal of this study was to compare how pregnancy and estrogen treatment affect ELP identified in Rag1/GFP reporter mice. The aim was to better characterize the most proximal event in lymphocyte formation that might be hormone regulated. Two strategies were then used to identify stromal cell genes that were both estrogen regulated and associated with inability to support B lymphopoiesis. We found that sFRP1 was not only up-regulated in estrogen-treated mice, but capable of selectively suppressing lymphocyte formation. Moreover, the protein stabilized β-catenin in hematopoietic progenitors without exogenous Wnt. The findings suggest a functional relationship between sex steroids and Wnt signaling in the regulation of initial stages of lymphopoiesis.

Materials and Methods

Animals, cell and compound sources

Rag1/GFP knockin mice have been described (10, 30, 31). BALB/c mice were obtained at 5 wk of age from Japan CLEA. Human mesenchymal cells (MC) derived from fetal BM (fetal MC) were established from anonymous tissues obtained from the Anatomic Gift Foundation. Adult human mesenchymal stem cells (adult MSC) were obtained from BioWhittaker. ERα and ERβ knockout mice have been previously described (32, 33). BMS2 and OP42, murine BM stromal lines, were maintained in 10% FCS-containing DMEM (high glucose) or α-MEM, respectively. Propyl pyrazole triol (34) and ERB-041 (35) were obtained from Wyeth-Ayerst Phar-

maceuticals. Recombinant sFRP1–4 and Wnt3a were obtained from R&D Systems.

Antibodies

FITC anti-Mac1 (M1/70), Gr-1 (Ly-6G; RB6-8C5), erythroid (TER-119), CD8a (53-6.7), CD19 (1D3), CD45R/B220 (RA3/6B2), and CD34 (RAM34) Abs; PE-anti-Mac1, CD45R/B220, CD19, c-kit (2B8), Sca1 (Ly6A/E; D7), and IL-7Rα (SB/199.1) Abs; biotin anti-Mac1, Gr-1, CD45R/B220, erythroid, and CD3 (145-2C11) Abs were obtained from BD Biosciences. Allophycocyanin anti-CD45R/B220 and c-kit Abs were purchased from eBioscience. Purified anti-Mac1, Gr-1, erythroid, CD3 (145-2C11), CD45RA (14.8), and CD19 Abs from BD Bioscience were used for depleting Lin$^+$ cells, followed by incubation with goat anti-rat IgG-coated magnetic beads (Miltenyi Biotec). A rabbit polyclonal Ab to sFRP1 (ab4193) and an anti-β-catenin Ab (14) were from Abcam and BD Biosciences, respectively.

Flow cytometry and cell sorting

Cells were stained with Abs indicated in each figure, and analyzed with FACSCalibur or FACSaria. Sorting of Lin$^-$ c-kit^{high} or Lin$^-$ c-kit^{low} cells from BALB/c mice was performed, as previously described (12). In some experiments, cells from Rag1/GFP heterozygotes were used to isolate Lin$^-$ IL-7Rα^- c-kit^{high} Sca1$^+$ Rag1/GFP$^-$ (HSC-enriched) or Lin$^-$ IL-7Rα^- c-kit^{high} Sca1$^+$ Rag1/GFP$^+$ (ELP) cells. Common lymphoid progenitors (CLP) (Lin$^-$ IL-7Rα^+ c-kit^{low} Sca1$^{low/-}$) and common myeloid progenitors (Lin$^-$ IL-7Rα^- c-kit^{high} Sca1$^-$ CD34$^+$ FcγRII/III^{low}) were sorted from C57BL/6 mice, as described (24, 36, 37).

Differential display PCR

Total RNA from fetal MC and adult MSC was first digested with 10 U of DNase I. The differential display PCR was performed using the RNAimage Kit (GeneHunter). Three different reverse-transcription reactions were conducted on 0.2 μg of DNA-free RNA using a 1- base anchor oligo(dT) primer, H-T11A, H-T11G, H-T11C (where H = AAGC) to generate different fractions of cDNAs. PCR were then performed on 1:10 aliquots of the reverse-transcription mixtures using the same anchor oligo(dT) primer (3-primer) and a 13-mer primer that contained a 7-base arbitrary sequence (5-primer). The sequences of differentially expressed cDNAs were determined using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Gene arrays

BMS2 and ERα-deficient stromal cells were treated with 10^{-7} M estrogen for 24 h, and mRNA was extracted. Gene Discovery Array Mouse version 1.1 (Incyte Genomics) blots were probed according to manufacturer's instructions. Briefly, mRNA was bound and eluted from oligo(dT) cellulose, and 2.5 μg of each mRNA was labeled during reverse transcription using [α-^{33}P]dCTP (2000–4000 Ci/mmol). Following hybridization to probes, arrays were exposed and imaged with a Molecular Dynamics STORM PhosphorImager. The gel files were sent to Incyte Genomics for analysis.

Stromal cell-free lymphocyte culture

Sorted cells were cultured in 24-well culture plates (3000–5000 cells/well) with SF-03 medium (Sanko-junyaku) containing 1% FCS, 50 μM 2-ME, 2 mM L-glutamine, 1 ng/ml murine rIL-7, 100 ng/ml murine flt-3 ligand, and 20 ng/ml recombinant murine stem cell factor, and fed every 4 days. At the end of culture, cells were counted and analyzed by flow cytometry.

Immunohistochemical staining

Tissues were fixed with 10% buffered formalin and embedded in paraffin. SFRP1 immunohistochemical staining was performed using a three-step immuno-alkaline phosphatase method and alkaline phosphatase-labeled streptavidin (DakoCytomation). Rabbit Ig (DakoCytomation) was used as negative control. The staining was performed in PBS containing 1% of BSA in the presence or absence of 10 μg/ml sFRP1.

Cell cycle and apoptosis

Sorted Lin$^-$ c-kit^{high} cells were cultured in stromal-free lymphocyte culture conditions for 24–36 h. At the end of culture, the cells were labeled with 10 μM BrdU for 45 min using the BD Biosciences BrdU labeling system. The cells were fixed for 10 min at room temperature in 4% paraformaldehyde/PBS, permeabilized, and then stained with FITC-conjugated anti-BrdU and 7-aminoactinomycin D. Apoptotic events were identified as cells with subdiploid DNA content.
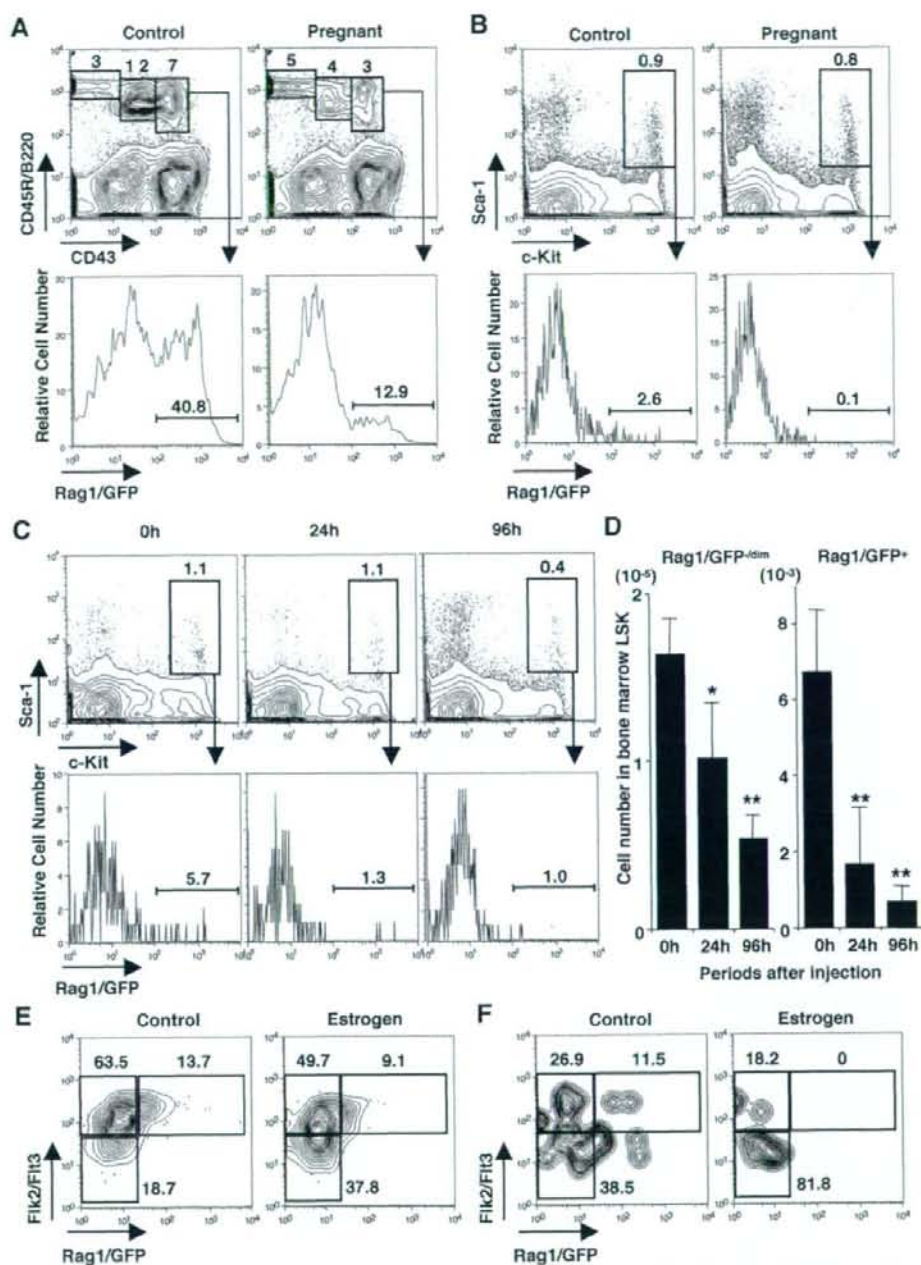
FIGURE 1. Early stages of B lymphopoiesis are blocked by pregnancy and estrogen treatment. BM cells of control mice or pregnant Rag1/GFP heterozygotes were stained with the indicated Ab and analyzed in flow cytometry. Whole BM cells were stained with CD45R/B220 and CD43 (*A, upper*), and Lin⁻ cells were gated and resolved with *c-kit* and *Sca*1 staining (*B, upper*). The CD45R/B220⁺ CD43^high fraction and the LSK fraction were further resolved with Rag-1/GFP intensity (*A* and *B, lower*). The numbers in each panel are percentages of the gated population. The figures represent data obtained on day 19.5 of gestation. Rag1/GFP heterozygous males were i.p. given a single injection of estrogen (1 mg/mouse) and analyzed at the indicated time after injection. BM Lin⁻ cells were resolved with *c-kit* and *Sca*1 (*C, upper*), and then the LSK fraction was further resolved according to Rag1/GFP intensity (*C, lower*). Cell numbers in the indicated populations were calculated (*D*). The data show mean ± SD values from four mice in each group, and the figures are representative of two independent trials. Significant differences from control (0 h) values are indicated by an asterisk ($p < 0.05$) or two asterisks ($p < 0.01$). *E*, BM LSK cells of estrogen-injected (at 96 h after injection) or control mice were subdivided according to the expression of Flk2/Flt3 and Rag1/GFP. Splenic LSK cells of the same mice were also evaluated (*F*).
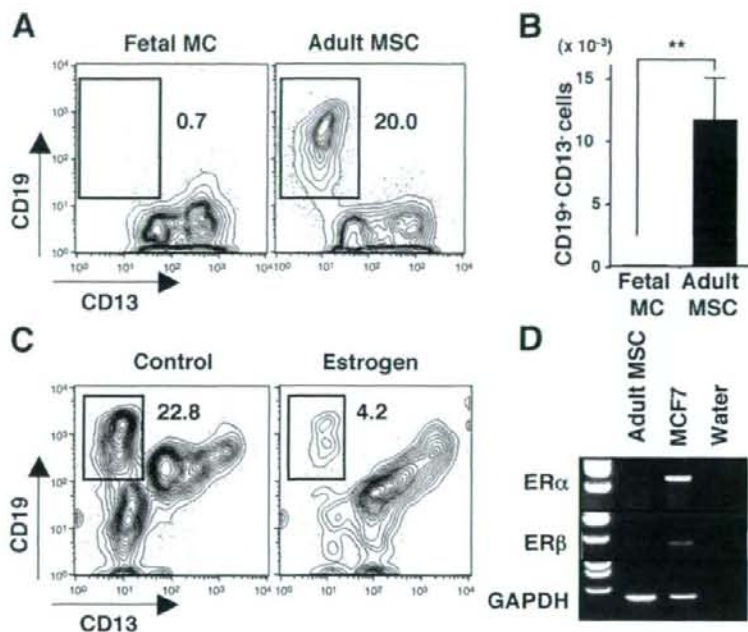
FIGURE 2. Establishment of two types of stromal cocultures that differ in lymphopoiesis-supporting capability. CD34⁺CD38⁻ human hematopoietic progenitors were cultured on fetal MC or adult MSC, and the output of CD19⁺ cells was evaluated (A and B). The figures represent three independent experiments. Influence of estrogen on the cell growth from human CD34⁺CD38⁻ progenitors was tested in the coculture using adult MSC (C). RT-PCR was used to amplify transcripts for ERα and β in adult MSC (D). MCF7, a human breast cancer line, was used as a positive control.

RT-PCR and quantitative real-time PCR

Total RNA was prepared from sorted cells and subjected to the cDNA synthesis using ThermoScript RT-PCR System (Invitrogen). PCR used a combination of ampli-*Taq*DNA polymerase (Takara Shuzo) and TaqStart Ab (BD Clontech) at 40 cycles for ERα and β, or 35 cycles for GAPDH. Real-time PCR analyses were performed using SYBR Green Mater Mix (Takara Shuzo). The thermal cycling conditions for the real-time PCR were 5 min at 95°C to active SYBR Ex *Taq*, followed by 40 cycles of denaturation, annealing, and extension. The mean number of cycles to the threshold of fluorescence detection was calculated for each sample, and β-actin expression was quantified to normalize the amount of cDNA in each sample. Semiquantitative RT-PCR was performed for Frizzled gene expression, as described (9). Primers for all of these reactions are available on request.

Statistical analyses

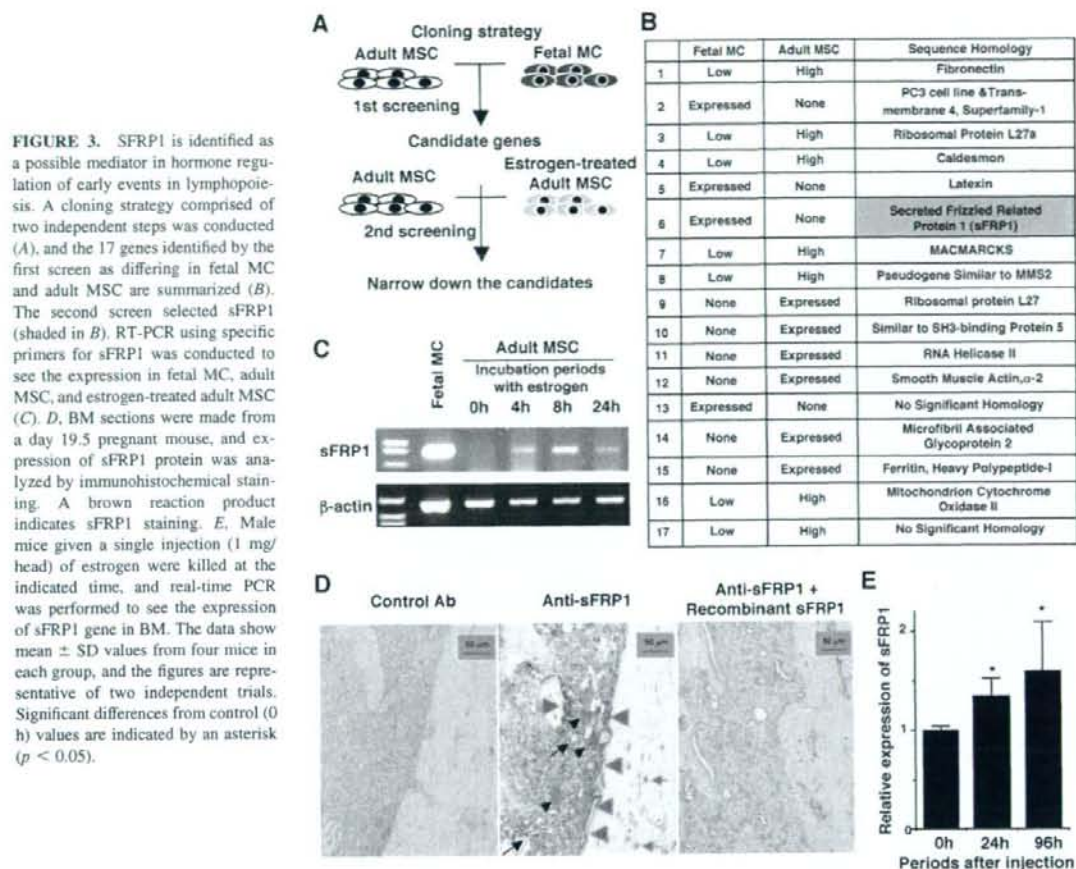Values of *p* were calculated by using unpaired Student's *t* test.

Results

Pregnancy and estrogen inhibit very early steps of lymphopoiesis

Previous studies have demonstrated that lymphocyte differentiation is strongly suppressed during pregnancy (38, 39). However, high resolution analysis of the earliest events in lymphopoiesis had not been performed. Therefore, we used Rag1/GFP heterozygous females at 14.5–19.5 days of gestation and compared their BM with nonpregnant females of the same age. Profiles of CD43 and CD45R/B220 expression showed that the CD43ʰⁱᵍʰCD45R/B220⁺ (pro-B-enriched) and CD43ˡᵒʷCD45R/B220⁺ (pre-B-enriched) fractions were preferentially depleted during pregnancy (Fig. 1A). Progenitors in the CD43ʰⁱᵍʰCD45R/B220⁺ fraction with clear expression of Rag1/GFP were particularly suppressed (Fig. 1A). This reporter system has been useful for identifying primitive Rag1 expressing ELP among the Lin⁻ Sca1⁺ c-kitʰⁱᵍʰ (LSK) fraction (10, 30). ELP were nearly absent from the BM of pregnant mice (Fig. 1B).

We previously demonstrated that estrogen, one of the pregnancy-related steroids, selectively suppressed Flk2⁺ CD27⁺ progenitors capable of generating B and/or T lineage cells among the LSK fraction (9). To allow comparison with the above results with pregnant mice, these experiments were repeated using Rag1/GFP reporter mice. Heterozygous males received single i.p. injections of estrogen, and changes in lymphopoiesis were monitored at intervals over the following 96 h. Decreases in Rag1/GFP-expressing cells in the LSK fraction became evident as early as 24 h after injection, and total numbers of Rag1⁺ ELP were reduced 10-fold by 96 h, whereas there were less severe depletions in total Rag1/GFP⁻/ᵈⁱᵐ LSK cells (Fig. 1, C and D). Thus, pregnancy and estrogen treatment are similar in preferentially depleting the earliest known stages of lymphopoiesis in BM. To more precisely analyze proportional change in the LSK fraction, we combined anti-Flk2/Flt3 Ab with Rag1/GFP expression because Flk2⁺ LSK cells were known as lymphoid-primed MPP (4). Indeed, the Flk2⁺ LSK contained Rag1⁺ ELP as a subpopulation. We found that Flk2⁺ Rag1⁻ as well as Flk2⁺ Rag1⁺ cells were sensitive to estrogen, whereas the Flk2⁻ Rag1⁻ LSK fraction was sustained (Fig. 1E). Of note, no increase of Rag1/GFP⁺ LSK cells was observed in spleen, peripheral blood, or thymi (Fig. 1F, and data not shown). In addition, numbers of CFU IL-7, corresponding to IL-7-responding pro/pre-B cells, also decreased with slower kinetics (data not shown). These results suggested that mobilization or rapid differentiation was an unlikely explanation for the depletion of ELP.

Human lymphopoiesis is hormone regulated

Molecular requirements for human B cell formation are not well understood, and the process is less efficient in culture than with murine lymphoid progenitors (40). However, B cells slowly emerge when human cells are cocultured with murine stromal cells (41), and we obtained better results with adult MSC (Fig. 2, A and B). That is, substantial numbers of CD19⁺CD13⁻ B lineage cells

FIGURE 3. SFRP1 is identified as a possible mediator in hormone regulation of early events in lymphopoiesis. A cloning strategy comprised of two independent steps was conducted (A), and the 17 genes identified by the first screen as differing in fetal MC and adult MSC are summarized (B). The second screen selected sFRP1 (shaded in B). RT-PCR using specific primers for sFRP1 was conducted to see the expression in fetal MC, adult MSC, and estrogen-treated adult MSC (C). D, BM sections were made from a day 19.5 pregnant mouse, and expression of sFRP1 protein was analyzed by immunohistochemical staining. A brown reaction product indicates sFRP1 staining. E, Male mice given a single injection (1 mg/head) of estrogen were killed at the indicated time, and real-time PCR was performed to see the expression of sFRP1 gene in BM. The data show mean ± SD values from four mice in each group, and the figures are representative of two independent trials. Significant differences from control (0 h) values are indicated by an asterisk ($p < 0.05$).

A Cloning strategy

B

	Fetal MC	Adult MSC	Sequence Homology
1	Low	High	Fibronectin
2	Expressed	None	PC3 cell line &Trans-membrane 4, Superfamily-1
3	Low	High	Ribosomal Protein L27a
4	Low	High	Caldesmon
5	Expressed	None	Latexin
6	Expressed	None	Secreted Frizzled Related Protein 1 (sFRP1)
7	Low	High	MACMARCKS
8	Low	High	Pseudogene Similar to MMS2
9	None	Expressed	Ribosomal protein L27
10	None	Expressed	Similar to SH3-binding Protein 5
11	None	Expressed	RNA Helicase II
12	None	Expressed	Smooth Muscle Actin,α-2
13	Expressed	None	No Significant Homology
14	None	Expressed	Microfibril Associated Glycoprotein 2
15	None	Expressed	Ferritin, Heavy Polypeptide-I
16	Low	High	Mitochondrion Cytochrome Oxidase II
17	Low	High	No Significant Homology

were produced from umbilical cord blood CD34$^+$CD38$^-$ cells within 4 wk of culture without any exogenous cytokines. In contrast, fetal MC did not support B lymphopoiesis.

Importantly, the output of CD19$^+$ cells on adult MSC decreased substantially when estrogen was added to the cultures (Fig. 2C). Culture studies done with murine stromal cells and lymphoid progenitors suggest that both are potential targets for hormone regulation (12, 14). Transcripts for ERα and ERβ were detected by RT-PCR in the MSC (Fig. 2D). Human cord blood progenitors do not generate lymphocytes in stromal cell-free cultures, but do not themselves express ER (11). We conclude that at least one hormone-sensitive human MC can support formation of human B lineage lymphocytes.

SFRPs are candidate inhibitors of lymphopoiesis

The focus of our next experiments was on identifying molecular mechanisms involved in estrogen action on lymphopoiesis. Our previous studies demonstrated that estrogen can directly suppress B lineage progenitors (12). It inhibited production of CD45R/B220$^+$ CD19$^+$ B lineage lymphocytes from early progenitors in stromal cell-free, serum-free culture, but accumulation of CD45R/B220$^+$ CD19$^-$ cells was observed (12). The results shown in Fig. 1 demonstrate that estrogen treatment and normal pregnancy affect lymphopoiesis before acquisition of CD45R/B220. In addition, as

shown in Fig. 2, estrogen suppressed human B cell growth in MSC cocultures from cord blood progenitors that do not express ER (11). Thus, the focus of the present experiments was on identifying mechanisms involving BM stromal cells.

Stromal cells might fail to support human B lymphopoiesis because of their inability to produce requisite molecules, and a differential display PCR method suggested that a number are preferentially made by adult MSC (Fig. 3, A and B). However, there was no evidence to implicate any in lymphopoiesis, and we considered the alternative possibility. That is, fetal marrow stromal cells might elaborate a suppressive substance, as was previously found to be the case for one murine stromal cell subclone (42). SFRP1 had those characteristics, and interest in this molecule increased further when RT-PCR with its specific primers confirmed that the transcripts were induced by treatment of adult MSC with estrogen (Fig. 3C). Immunohistochemical analysis showed that bone-lining osteoblast-like stromal cells and osteoclasts expressed sFRP1 protein (Fig. 3D, large orange arrow). Although most hematopoietic cells appeared negative, endothelial cells (small black arrow), osteocytes (small orange arrow), and megakaryocytes (black arrowhead) had positive staining. Moreover, sFRP4 as well as sFRP1 mRNA were detectable in unseparated BM cells and elevated in day 14.5 pregnant mice (sFRP1: first experiment, 120%, and second experiment, 560% of control levels; sFRP4: first experiment,
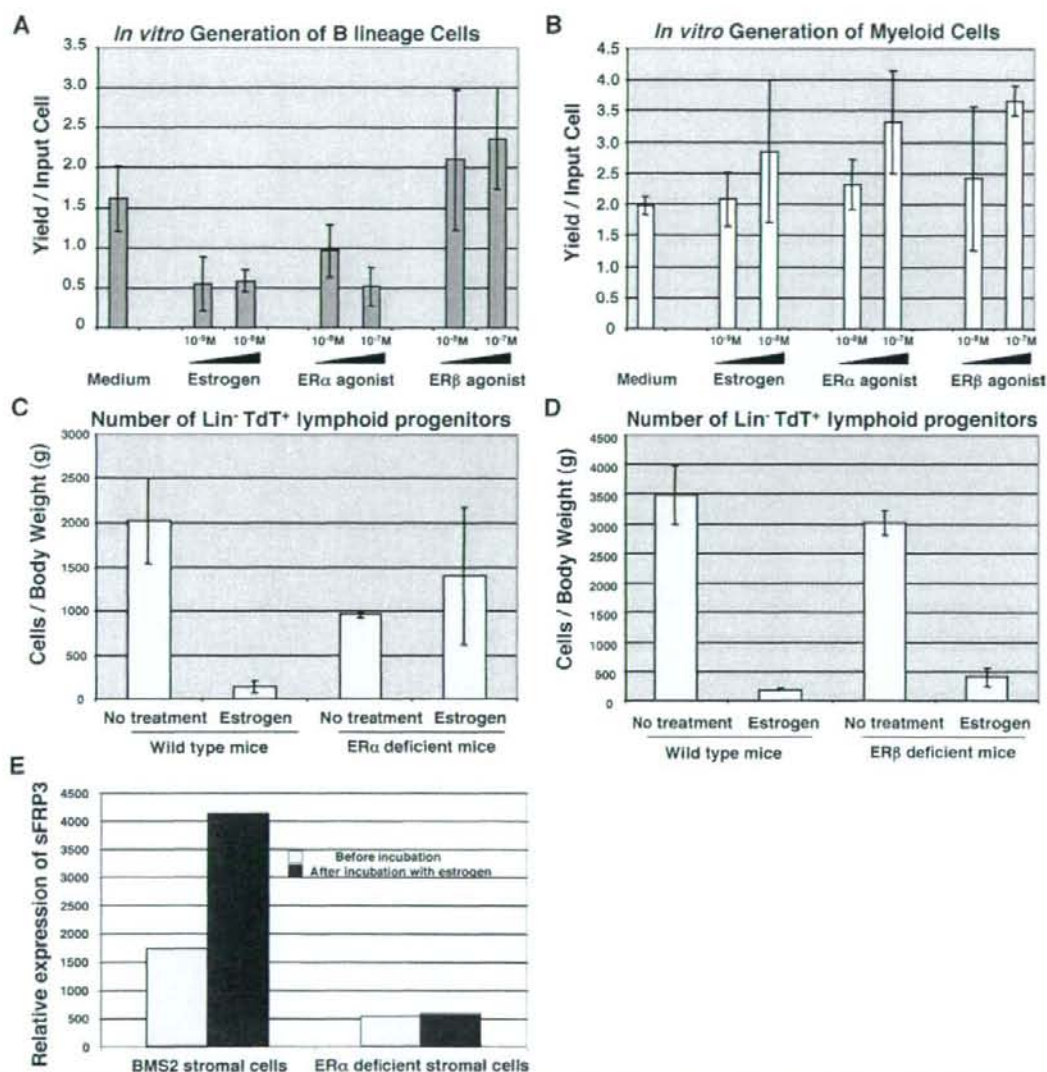
FIGURE 4. Estrogen induces the sFRP family via ERα. *A* and *B*, ERα-selective, but not ERβ-selective agonists inhibited in vitro generation of B lymphocytes. Lin⁻ BM cells were cultured in stromal cell-free conditions for 7 days. Each culture contained estrogen, ERα-selective agonist (propyl pyrazole triol), or ERβ-selective agonist (ERB-041) in various concentrations. At the end of culture, numbers of B lineage (CD19⁺) cells (*A*) and myeloid (Mac1⁺) cells were evaluated. Averages and SD of triplicate cultures are shown. *C* and *D*, Estrogen suppresses early pro-B cells in vivo via ERα. ERα-deficient, ERβ-deficient, or control mice were injected s.c. with slow-release estrogen containing pellets. One week after injection, BM cells were harvested and subjected to flow cytometry to determine Lin⁻ TdT⁺ cell numbers. *E*, BMS2 or ERα⁻/⁻ stromal cells were exposed to estrogen, and gene arrays were performed. The sFRP3 mRNA results are presented as relative levels of expression.

170%, and second experiment, 180% of control levels). Also, a single injection of estrogen significantly elevated sFRP1 transcripts to 150–160% of steady-state levels (Fig. 3*E*).

Independent lines of investigation with murine cells had also suggested that sFRPs might participate in regulation of lymphopoiesis. Some evidence suggests ERα mediates estrogen suppression of lymphopoiesis (13, 43). Indeed, an ERα-selective, but not an ERβ-selective agonist suppressed B lineage growth in culture (Fig. 4, *A* and *B*). Lymphoid progenitors were less abundant in ERα⁻/⁻ mice than in normal animals and resistant to estrogen

treatment (Fig. 4*C*). This is in contrast to ERβ⁻/⁻ mice, in which lymphopoiesis was normally suppressed by hormone treatment (Fig. 4*D*). Our search was then focused on genes that might be selectively affected by signals delivered via ERα.

Although a now obsolete membrane gene array method was used, some 260 genes were up-regulated by at least 2-fold when BMS2 stromal cells were exposed to estrogen, and an additional 70 were suppressed to at least that degree. BMS2 is an extensively studied stromal cell line derived from normal mice (44). Estrogen increased expression of ~85 genes in stromal cells established
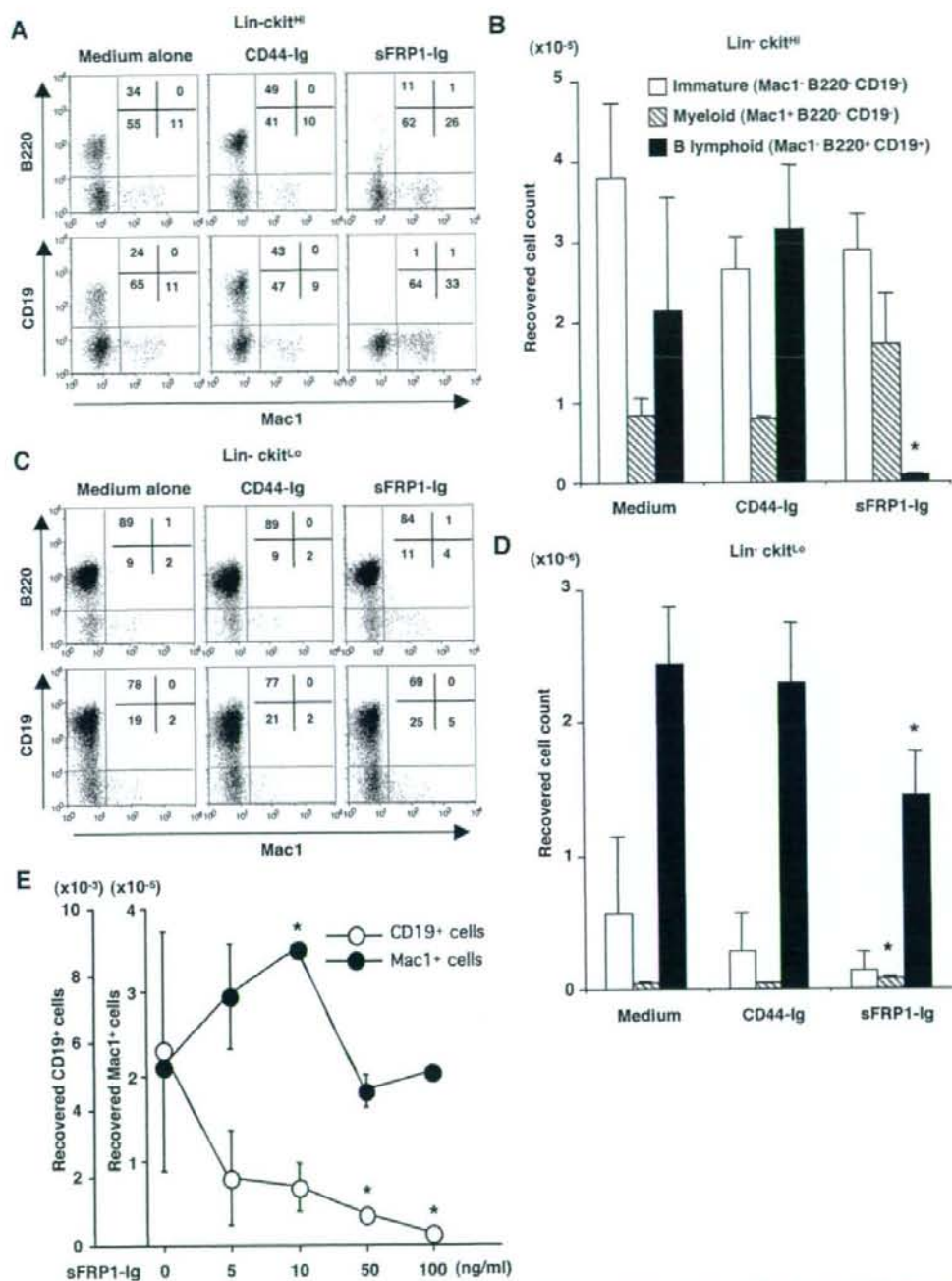
FIGURE 5. SFRP1 inhibits early B lymphopoiesis in culture. Sorted Lin$^-$ c-kit^{high} (*A* and *B*) or Lin$^-$ c-kit^{low} cells (*C* and *D*) were cultured in stromal-free conditions with medium alone, CD44-Ig, or sFRP1-Ig, and growth of myeloid and B lineage in each culture was evaluated. The percentages of four fractions are shown in boxes. The absolute number of undifferentiated cells (Mac1$^-$ CD45R/B220$^-$ CD19$^-$), myeloid cells (Mac1$^+$ CD45R/B220$^-$ CD19$^-$), and B lineage cells (Mac1$^-$ CD45R/B220$^+$ CD19$^+$) recovered from culture of Lin$^-$ c-kit^{high} (*C*) or Lin$^-$ c-kit^{low} cells (*D*) was calculated. The data represent the mean ± SD values from triplicate cultures. Significant differences from control (CD44-Ig) values are indicated by an asterisk (*p* < 0.05). Data represent one of six similar experiments. *E*, Lin$^-$ c-kit^{high} cells (3000 cells/well) were cultured in stromal-free condition with the indicated concentration of sFRP1-Ig. Absolute numbers of CD19$^+$ cells (○) or Mac1$^+$ cells (●) recovered were calculated and plotted. Significant differences from control values (at sFRP1-Ig, 0 ng/ml) are indicated by an asterisk (*p* < 0.05).

from marrow of $ER\alpha^{-/-}$ mice (data not shown). Comparison of the two sets of results suggested that ~160 genes are ERα dependent, and thus, potential candidates for hormone-mediated regulation of lymphopoiesis. We found that sFRP3 was among those genes and strongly induced in estrogen-treated BMS2 cells (Fig. 4E).

Further analysis revealed that transcripts for sFRP1, sFRP2, and sFRP3 were elevated when a third stromal cell line, OP42, was exposed to estrogen, whereas sFRP4 expression was constitutive. We then asked whether estrogen directly induced sFRPs in hematopoietic cells. Another gene array involving the Lin^- $c-kit^{high}$ $Sca1^+$ fraction of normal BM also identified sFRP1, sFRP2, and sFRP4 as estrogen-inducible genes (data not shown). Thus, members of the sFRP family are normally present in BM and probably up-regulated in multiple cell types in response to estrogen.

### Selective suppression of early stages of lymphopoiesis by sFRP1

The above results suggested that sFRPs could be hormone-inducible, negative regulators of lymphopoiesis. In particular, sFRP1 might account for the fact that one stromal line supports B lymphopoiesis in the absence, but not in the presence of estrogen. To test this notion, substantial amounts of stable sFRP1-Ig fusion protein, along with a control CD44-Ig, were prepared. They were first added at 100 ng/ml to stromal cell-free cultures of BALB/c mouse BM to determine whether there was direct biological activity on lymphoid progenitors. Starting populations used in these experiments included stem cell/ELP-enriched Lin^- $c-kit^{high}$, as well as prolymphocyte/CLP-enriched Lin^- $c-kit^{low}$ subsets. After 10–11 days of culture with just medium or CD44-Ig, the Lin^- $c-kit^{high}$ fraction produced 20–50% $CD45R/B220^+$ $CD19^+$ cells (Fig. 5A). In striking contrast, lymphocytes represented only 1% of cells recovered from cultures containing sFRP-Ig. Undifferentiated $CD11b/Mac1^-$ $B220^-$ $CD19^-$ and $CD11b/Mac1^+$ $B220^-$ $CD19^-$ myeloid cells were also abundant in this set of cultures. It is significant that sFRP1-Ig had no influence on percentages or absolute numbers of these nonlymphoid cells (Fig. 5, A and B). Lymphocytes predominated in cultures initiated with the Lin^- $c-kit^{low}$ fraction, and their numbers were reduced to a lesser, but still significant degree by sFRP1-Ig (Fig. 5, C and D).

We then performed a dose-response analysis of sFRP1-Ig in the culture of Lin^- $c-kit^{high}$ cells to see whether different outcomes would be obtained at different doses. The suppressive effect on $CD19^+$ lineage was dose dependent (Fig. 5E). Essentially, similar results were obtained when human rsFRP1 was used instead of the fusion protein (see below), showing that the suppression was specific to sFRP1. Proportions and absolute numbers of $Mac1^+$ cells were not significantly reduced, but rather increased by 5–10 ng/ml protein (Fig. 5E).

In separate experiments, sFRP1-Ig had no influence on IL-7-dependent clonal proliferation of pre-B cells (data not shown). Furthermore, there was no effect on myeloid-erythroid progenitors in methylcellulose assays (Fig. 6). Collectively, these results suggested that sFRP1 is a potential negative regulator of B lymphopoiesis, and one that is preferentially active on very primitive cells.

### Stabilization of β-catenin by sFRP1 in hematopoietic progenitors

The sFRPs were originally described as inhibitors for Wnt by blocking their interactions with Frizzled receptors (15). More recently, sFRPs have been shown to function in some circumstances in Wnt-independent fashion (19, 20). Although we found that sFRP1 directly influenced lymphocyte growth in stromal-free cultures, hematopoietic cells have some capacity to produce their own Wnt (45). Wnt can be divided into functionally distinct classes that
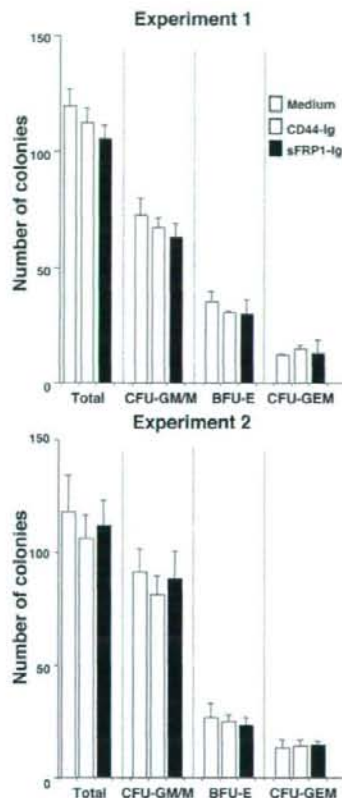
FIGURE 6. SFRP1-Ig does not affect myeloerythroid colony formation in methylcellulose cultures. Five hundred Lin^- $c-kit^{high}$ cells were cultured in IMDM-based methylcellulose medium supplemented with 50 ng/ml recombinant murine stem cell factor, 10 ng/ml murine rIL-3, 10 ng/ml human rIL-6, and 3 U/ml recombinant human erythropoietin (Methocult GF 3434; StemCell Technologies) with medium alone, 100 ng/ml CD44-Ig, or 100 ng/ml sFRP1-Ig. After 9 days, colonies were enumerated and classified as CFU-GM/M, BFU-E, or CFU-GEM(M) according to shape and color under an inverted microscope.

can use different signal transduction mechanisms (46). Therefore, we compared commercial sFRP1 and Wnt3a preparations for a possible influence on lymphocyte production. Because Lin^- $c-kit^{high}$ cells were particularly sensitive in the experiments described above, these were used to initiate stromal cell-free cultures. Interestingly, the two proteins showed similar results. A quantity amounting to 50 nM sFRP1 or Wnt3a reduced the yields of $Mac1^-$ $CD19^+$ $CD45R/B220^+$ B lineage cells by 42 and 33%, respectively (Fig. 7A). The suppressive effect was also evident when the proteins were used at 5–10 nM (data not shown). The suppression was unlikely due to the induction of apoptosis because neither cell viability nor proportion of cells with subdiploid DNA content was significantly increased in the presence of sFRP1 through the culture period (Fig. 7B, and data not shown). Similar suppression for B lineage was observed when the culture was initiated with sFRP2, sFRP3, or sFRP4 (data not shown). Of note, sFRP1 and sFRP4 transcripts were at least as abundant in fetal liver as in adult BM (data not shown). However, generation of B lineage cells from E14.5 fetal liver

FIGURE 7. SFRP1 stimulates the β-catenin pathway in hematopoietic progenitors. *A,* Lin⁻ c-*kit*^high cells were cultured in stromal-free condition containing 50 nM sFRP1 or Wnt3a, and the recovered cells were classified according to their surface phenotype. The data summarize four independent experiments, showing mean ± SD values of percentage of control. The normalized control (medium) value is shown at 100% with a dotted line. Significant differences ($p < 0.05$, indicated by an asterisk) from control value were constantly observed regarding B lymphoid cells in the four trials. *B,* Cell cycle and apoptosis analyses were performed with Lin⁻ c-*kit*^high cells after 36 h of stromal-free culture. Percentages of cells in the subdiploid fraction and S phase are shown in each panel. *C,* SFRP1 and Wnt3a were added to the same cultures at the indicated concentration to test their mutual influence. Significant differences from control (0 nM Wnt3a, 0 nM sFRP1) values are indicated by an asterisk ($p < 0.05$). Similar results were obtained in two independent experiments. *D,* Lin⁻ c-*kit*^high cells were cultured in stromal-free condition containing 5 nM BIO, a glycogen synthase kinase-3-specific inhibitor, and the recovered cells were classified. *E,* Cytospin preparations of Lin⁻ c-*kit*^high cells incubated with 50 nM sFRP1 for 24 h were subjected to immunohistochemistry with an anti-β-catenin Ab. *F,* LSK cells were incubated with 50 nM sFRP1, Wnt3a, or BIO for 24 h, respectively. Then 1×10^4 cells of each were subjected to Western blotting for β-catenin. The membrane was reblotted with an anti-actin Ab. The data are representative of two independent experiments.
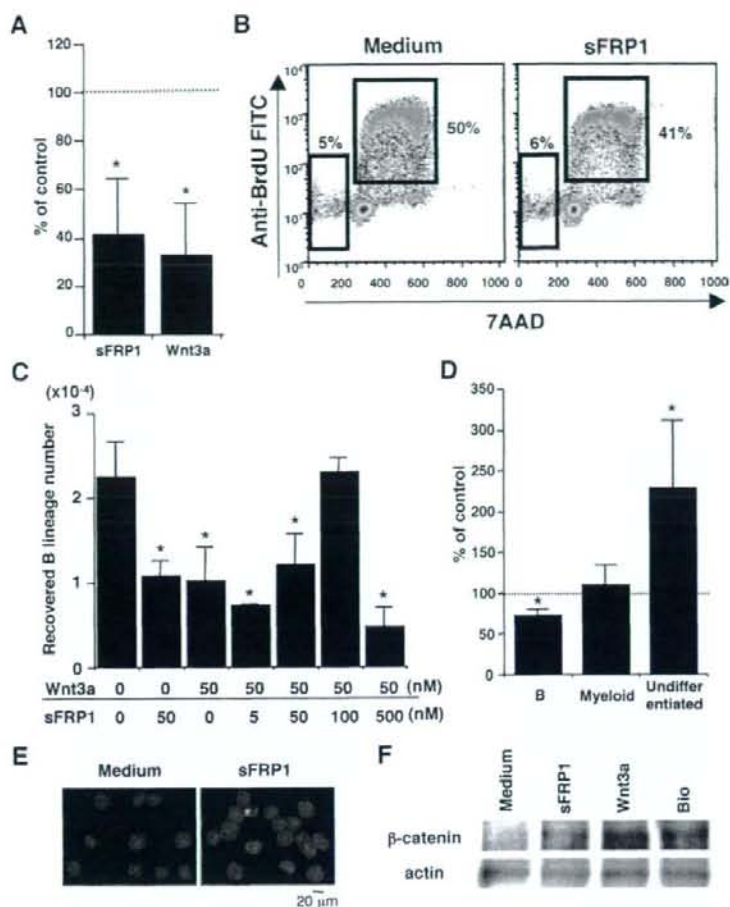
Lin (TER-119, Gr-1)⁻ c-*kit*^high Sca1⁺ cells was not suppressed by either sFRP1-Ig or sFRP1 (data not shown).

Similar cultures were then initiated in which sFRP1 and Wnt3a were added together in different ratios (Fig. 7C). As expected, each reduced B lymphopoiesis by ∼60% when added alone. However, there was mutual interference when 100 nM sFRP1 was added to cultures containing 50 nM Wnt3a. These results demonstrate that whereas each of these ligands can suppress lymphocyte formation, they cross-antagonize each other when present at the same time.

The best studied of major signaling pathways attributed to Wnt involves stabilization of β-catenin and its subsequent accumulation in the nucleus (18). Recent reports showed that artificial β-catenin expression strongly inhibited lymphopoiesis in vitro and in vivo (24, 47, 48). Therefore, we wondered whether sFRP1 would stimulate canonical Wnt signaling, because it appears to act independently of Wnt on lymphoid progenitors. As a positive control, we used 6-bromoindirubin-37-oxime (BIO), a specific pharmacological inhibitor of glycogen synthase kinase-3. B lymphopoiesis was suppressed by 70% of control when BIO was added to BM cultures at 5 nM corresponding with its IC₅₀ (Fig. 7D). In contrast, numbers of Mac1⁺ myeloid cells were unchanged, and cells with an immature phenotype expanded by more than 2-fold (Fig. 7D).

We then sought evidence for β-catenin stabilization in Lin⁻ c-*kit*⁺ cells incubated in the presence of those proteins for 24 h. As a positive control, we again used 50 nM BIO. Fluorescence microscopy revealed that some of the sFRP1- or Wnt3a-treated Lin⁻ c-*kit*⁺ cells (20% of sFRP1 treated, 29% of Wnt3a treated in this experiment) showed cytoplasmic and nuclear accumulation of β-catenin, whereas cells treated with medium alone did not (Fig. 7E, and data not shown). Western blotting analyses were performed for highly enriched LSK cells. Fig. 7F showed that BIO, sFRP1, and Wnt3a all caused accumulation of β-catenin in treated cells.

Wnt receptors tend to be down-regulated as lymphopoiesis proceeds

The results shown above suggested that sFRP1 and Wnt3a can stimulate the β-catenin pathway and preferentially influence early steps in lymphopoiesis. To better understand this vulnerability, we examined the expression pattern of Frizzled receptors on stem and progenitor cells. Interestingly, HSC highly expressed seven of nine Frizzled receptors, which markedly declined as they differentiated to ELP (Fig. 8). Only two receptors, Frizzled 6 and 9, remained detectable in the lymphoid-restricted Lin⁻ c-*kit*^low Sca1^low IL-7Rα⁺ CLP. Most Frizzled receptors also declined with commitment to the myeloid lineage, but Frizzled 1 emerged on common
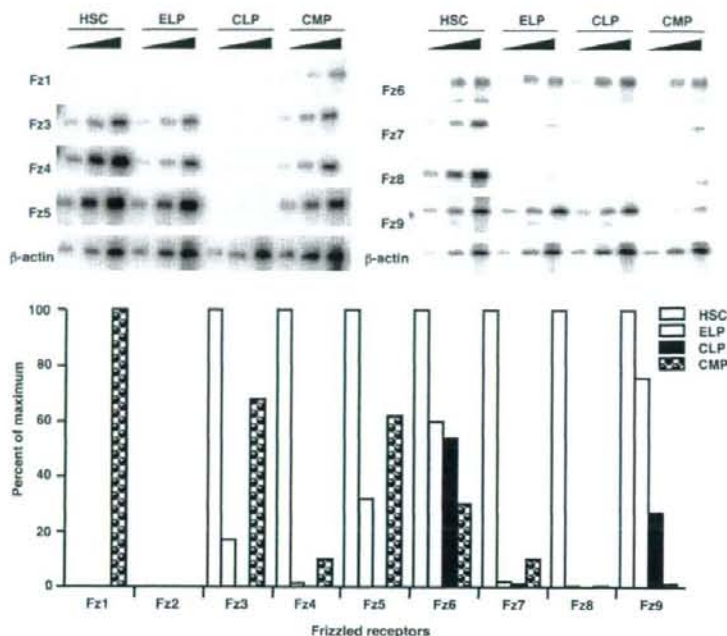
FIGURE 8. Frizzled receptors diminish along with lymphoid lineage specification. The indicated progenitors were isolated, and semiquantitative RT-PCR was conducted to amplify transcripts for the indicated Frizzled receptors in each population. The bar graphs represent the results of $[\alpha\text{-}^{32}P]dCTP$ incorporation on the linear parts of PCR amplification curves normalized according to β-actin expression.

myeloid progenitors. Also noteworthy is the fact that stem/progenitor cells expressed Frizzled 4, a receptor used by Wnt5a for canonical signaling (29). These results indicate how maturing lymphoid lineage cells might become less responsive to Wnt signaling as they differentiate.

Discussion

Sex steroids most likely contribute to normal steady-state regulation as well as pregnancy-related suppression of lymphopoiesis, but progress has been slow in identifying relevant molecular mechanisms. Not only are there multiple cellular targets for hormone action in BM, but many responsive genes. Lymphopoietic activity declines with senescence due to as yet unknown mechanisms, although involvement of age-related hormones has been speculated (49, 50). The present study focused on the hematopoietic microenvironment as represented by stromal cells, and a number of observations point to sFRP1 as one potential mediator of estrogen action. It was hormone inducible in stromal cells that normally support lymphopoiesis, and constitutive in ones that did not. Two recombinant forms of sFRP1 suppressed early stages in B lymphopoiesis while sparing nonlymphoid progenitors. It was similar in this respect to rWnt3a. These and other studies show that the activity of sFRP1 is context dependent, using the Wnt signaling pathway even when other Wnts are absent.

Much has been learned about founders of the immune system, and it is now possible to identify cells that initially transcribe lymphoid genes. These ELP are included in the rare $Lin^- Sca1^+ c\text{-}kit^{high} Flk2^+ CD27^+ Thy1.1^- VCAM\text{-}1^-$ subset of BM. Low-level expression of a human μ transgene, TdT, Rag1, and/or IL-7Rα are indications that these progenitors are primed for lymphopoiesis, and viable cells enriched on the basis of a Rag1 or an Ikaros reporter are highly potent in this regard (10, 51). The size of this population may be carefully regulated inasmuch as it supplies progenitors needed to replenish T, B, NK, and some dendritic cells. Our comparison of pregnant and estrogen-treated mice now implicates sex steroids in that process. Although $Rag1^+$ ELP appeared to be the most hormone sensitive of BM

cells, we also recorded reductions in a companion $Rag1^- Flk2^+$ population.

It has been repeatedly documented that the immune system is altered during pregnancy (52, 53). Substantial down-regulation in both T and B lymphopoiesis is also reported (38, 39), and estrogen is likely to play a pivotal role in changes related to maternal immunity. However, there has been little information regarding physiological changes in the earliest stages of lymphopoiesis. Our present data clearly described that the first step of lymphopoiesis in BM is particularly suppressed during pregnancy.

A major goal of this study was to identify molecules involved in the estrogen-induced down-regulation of early stages of lymphopoiesis. Our previous studies showed that the hormone has direct effect on lymphocyte formation in culture (12). However, this does not precisely replicate changes that occur in BM of pregnant or estrogen-treated animals. For example, CLP/prolymphocytes were particularly sensitive in stromal-free cultures, whereas dramatic changes in more primitive ELP were recorded in the in vivo experiments described above. Consequently, the emphasis of our present study was on the environment, and we exploited stromal cells to identify candidate hormone-regulated molecules.

Culture systems that support human B lymphopoiesis are inefficient (54), inspiring a search for better stromal cells. One was completely nonsupportive, whereas commercially available human MSC were more effective than murine stromal cells that are commonly used. These findings were reported in more detail elsewhere (55), and we only used them in this study as a means of further narrowing our search for hormone mediators. Importantly, sFRP1 was identified as a candidate regulator of lymphopoiesis via independent approaches.

Our previous findings suggested that estrogen induces suppressive factors for lymphopoiesis in stromal cells (14). Gene screening approaches are now highly efficient and yield a surfeit of candidates. Levels of at least 330 genes changed substantially when stromal cells were exposed to estrogen in culture. Although either

of the two known ER could be used, the list of candidate genes was reduced to ~160 when stromal cells prepared from $ER\alpha^{-/-}$ mice were stimulated. Several findings and our present results suggest that this receptor is important in regulation of lymphopoiesis (13, 43) (Fig. 4, A–D). SFRPs remained among the candidates that were markedly inducible in wild-type, but not in $ER\alpha^{-/-}$ stromal cells. Other genes were eliminated from consideration when we found that lymphopoiesis was normally suppressed in BM of the corresponding knockout mice. These included Fas, p53, p21, Nur77, and Bax (our unpublished observations).

Although sFRPs produced by estrogen-stimulated stromal cells can arrest the earliest stages of lymphopoiesis in BM, there may be additional hormone-dependent regulatory mechanisms. For example, estrogen might directly cause sFRP production in some hematopoietic cells. Rolink and colleagues (56) recently found 2-fold reductions in BM IL-7 mRNA during pregnancy. Residual IL-7-responding progenitor in pregnant mouse marrow expanded when exposed to high concentrations of this essential cytokine. However, pregnancy fluctuations in IL-7 would seem not to account for depletion of the most primitive lymphopoietic cells, because these ELP lack IL-7Rs (10).

Given their wide tissue distribution, sFRPs could have multiple roles (57). Transcripts for sFRP1 were substantially induced when stromal cells were exposed to estrogen. Stromal cells are rare among maturing blood cells in BM, but hormone treatment caused 1.5-fold increases in sFRP1 expression (Fig. 3E). Targeting of the sFRP1 gene resulted in increased trabecular bone formation (58). It is interesting that osteoblastic stromal cells that line trabecular bone can make sFRP1 (Fig. 3D) because they are thought to provide one niche for HSC (59, 60).

SFRP1 was originally isolated as an extracellular inhibitor for Wnt signaling that contains a CRD homologous to the putative Wnt-binding domain of Frizzled (61). Although several studies verified that sFRP1 is a Wnt antagonist, Üren et al. (62) also demonstrated a biphasic action of sFRP1, and at low concentrations it potentiates the activity of Wingless, a Wnt homologue in Drosophila. Our data (Fig. 7) are consistent with those reports and show that sFRP1 itself may potentially transduce Wnt-like signals in early hematopoietic progenitors when exogenous Wnt ligands are not provided.

Many of the functions ascribed to the five sFRPs relate to their interactions with Wnt, but there is accumulating evidence that they can signal independently of Wnt. For example, Bafico et al. (63) showed that the CRD of sFRP1 could directly bind to Frizzled. Although it was first speculated that the interaction created nonfunctional receptor complexes, later studies revealed that sFRP1 enhanced retinal neurogenesis via Frizzled 2, independently of canonical Wnt signaling (20). Our data indicate that a similar mechanism may be operable in hematopoietic cells. Although Frizzled 2 was not detected in hematopoietic progenitors, sFRP1 may interact with other Frizzled receptors, and directly affect early events in lymphoid differentiation. Interestingly, one early study showed that sFRP2, in contrast to sFRP1, stabilized β-catenin in MCF7 breast cancer cells and enabled the cells to resist TNF-induced apoptosis (64). Comparing expression patterns of Frizzled on MCF7 might provide a hint about which receptor is used by sFRP1 on hematopoietic progenitors, but there are obviously many possibilities.

It is controversial if and how members of the complicated Wnt family regulate hematopoiesis under normal conditions because it has been difficult to design loss of function approaches that could target the 19 Wnt ligands and multiple signaling pathways (65). That is also the case for the sFRP family, comprised of 5 members whose function might be redundant. Eliminating β-catenin is em-

bryonic lethal, but conditional targeting of that and the related γ-catenin genes in hematopoietic cells did not compromise HSC (66, 67). In contrast, there was evidence that this manipulation failed to block constitutive Wnt signaling (68). Furthermore, artificial β-catenin accumulation retards hematopoiesis, and sustained stimulation of this pathway leads to BM failure (47, 48). Wnt signals may normally contribute to stem cell integrity, because introduction of constitutively active β-catenin allowed multipotential cells to be expanded in culture and even made committed progenitors multipotential (22, 24, 25).

Our observations implicate the sFRP family as possible mediators in hormone regulation of the earliest events in lymphopoiesis. Myeloerythroid progenitors were unaffected by exposure to sFRP1 in culture, suggesting that it is similar to estrogen with respect to lineage specificity. However, further investigation of T, NK, and plasmacytoid dendritic lineages is needed to determine whether that is the case. The available information suggests that sFRP1 functions in a context-dependent fashion, acting as agonists or antagonists, depending on the presence of other ligands and particular receptors. Although the complexity of the Wnt family is intimidating, there is reason to believe that further study will lead to new therapeutic strategies for lymphoid malignancies.

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Disclosures

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References

1. Busslinger, M. 2004. Transcriptional control of early B cell development. Annu. Rev. Immunol. 22: 55–79.
2. Rothenberg, E. V., and T. Taghon. 2005. Molecular genetics of T cell development. Annu. Rev. Immunol. 23: 601–649.
3. Medina, K. L., J. M. Pongubala, K. L. Reddy, D. W. Lancki, R. Dekoter, M. Kieslinger, R. Grosschedl, and H. Singh. 2004. Assembling a gene regulatory network for specification of the B cell fate. Dev. Cell 7: 607–617.
4. Adolfsson, J., R. Månsson, N. Buza-Vidas, A. Hultquist, K. Liuba, C. T. Jensen, D. Bryder, L. Yang, O. J. Borge, L. A. Thoren, et al. 2005. Identification of Flt3⁺ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121: 295–306.
5. Nakano, T. 2003. Hematopoietic stem cells: generation and manipulation. Trends Immunol. 24: 589–594.
6. Kincade, P. W., K. L. Medina, G. Smithson, and D. C. Scott. 1994. Pregnancy: a clue to normal regulation of B lymphopoiesis. Immunol. Today 15: 539–544.
7. Smithson, G., W. G. Beamer, K. L. Shultz, S. W. Christianson, L. D. Shultz, and P. W. Kincade. 1994. Increased B lymphopoiesis in genetically sex steroid-deficient hypogonadal (hpg) mice. J. Exp. Med. 180: 717–720.
8. Medina, K. L., and P. W. Kincade. 1994. Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. Proc. Natl. Acad. Sci. USA 91: 5382–5386.
9. Medina, K. L., K. P. Garrett, L. F. Thompson, M. I. Rossi, K. J. Payne, and P. W. Kincade. 2001. Identification of very early lymphoid precursors in bone marrow and their regulation by estrogen. Nat. Immunol. 2: 718–724.
10. Igarashi, H., S. C. Gregory, T. Yokota, N. Sakaguchi, and P. W. Kincade. 2002. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity 17: 117–130.
11. Igarashi, H., T. Kouro, T. Yokota, P. C. Comp, and P. W. Kincade. 2001. Age and stage dependency of estrogen receptor expression by lymphocyte precursors. Proc. Natl. Acad. Sci. USA 98: 15131–15136.
12. Kouro, T., K. L. Medina, K. Oritani, and P. W. Kincade. 2001. Characteristics of early murine B-lymphocyte precursors and their direct sensitivity to negative regulators. Blood 97: 2708–2715.
13. Thurmond, T. S., F. G. Murante, J. E. Staples, A. E. Silverstone, K. S. Korach, and T. A. Gasiewicz. 2000. Role of estrogen receptor α in hematopoietic stem cell development and B lymphocyte maturation in the male mouse. Endocrinology 141: 2309–2318.
14. Smithson, G., K. Medina, I. Ponting, and P. W. Kincade. 1995. Estrogen suppresses stromal cell-dependent lymphopoiesis in culture. J. Immunol. 155: 3409–3417.

15. Kawano, Y., and R. Kypta. 2003. Secreted antagonists of the Wnt signalling pathway. *J. Cell Sci.* 116: 2627–2634.

16. Rattner, A., J. C. Hsieh, P. M. Smallwood, D. J. Gilbert, N. G. Copeland, N. A. Jenkins, and J. Nathans. 1997. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc. Natl. Acad. Sci. USA* 94: 2859–2863.

17. Lin, K., S. Wang, M. A. Julius, J. Kitajewski, M. Moos, Jr., and F. P. Luyten. 1997. The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. *Proc. Natl. Acad. Sci. USA* 94: 11196–11200.

18. Gordon, M. D., and R. Nusse. 2006. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J. Biol. Chem.* 281: 22429–22433.

19. Collavin, L., and M. W. Kirschner. 2003. The secreted Frizzled-related protein Sizzled functions as a negative feedback regulator of extreme ventral mesoderm. *Development* 130: 805–816.

20. Rodriguez, J., P. Esteve, C. Weinl, J. M. Ruiz, Y. Fermin, F. Trousse, A. Dwivedy, C. Holt, and P. Bovolenta. 2005. SFRP1 regulates the growth of retinal ganglion cell axons through the Fz2 receptor. *Nat. Neurosci.* 8: 1301–1309.

21. Dosen, G., E. Tenstad, M. K. Nygren, H. Stubberud, S. Funderud, and E. Rian. 2006. Wnt expression and canonical Wnt signaling in human bone marrow B lymphopoiesis. *BMC Immunol.* 7: 13.

22. Reya, T., A. W. Duncan, L. Ailles, J. Domen, D. C. Scherer, K. Willert, L. Hintz, R. Nusse, and I. L. Weissman. 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature* 423: 409–414.

23. Nelson, W. J., and R. Nusse. 2004. Convergence of Wnt, β-catenin, and cadherin pathways. *Science* 303: 1483–1487.

24. Baba, Y., K. P. Garrett, and P. W. Kincade. 2005. Constitutively active β-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. *Immunity* 23: 599–609.

25. Baba, Y., T. Yokota, H. Spits, K. P. Garrett, S. Hayashi, and P. W. Kincade. 2006. Constitutively active β-catenin promotes expansion of multipotent hematopoietic progenitors in culture. *J. Immunol.* 177: 2294–2303.

26. Liang, H., Q. Chen, A. H. Coles, S. J. Anderson, G. Pihan, A. Bradley, R. Gerstein, R. Jurecic, and S. N. Jones. 2003. Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. *Cancer Cells* 4: 349–360.

27. Malhotra, S., Y. Baba, K. P. Garrett, F. J. T. Staal, R. Gerstein, and P. W. Kincade. 2008. Contrasting responses of lymphoid progenitors to canonical and non-canonical Wnt signals. *J. Immunol.* 181: 3955–3964.

28. Topol, L., X. Jiang, H. Choi, L. Garrett-Beal, P. J. Carolan, and Y. Yang. 2003. Wnt-5a inhibits the canonical Wnt pathway by promoting GSK-3-independent β-catenin degradation. *J. Cell Biol.* 162: 899–908.

29. Mikels, A. J., and R. Nusse. 2006. Purified Wnt5a protein activates or inhibits β-catenin-TCF signaling depending on receptor context. *PLoS Biol.* 4: 0570–0582.

30. Yokota, T., T. Kouro, J. Hirose, H. Igarashi, K. P. Garrett, S. C. Gregory, N. Sakaguchi, J. J. T. Owen, and P. W. Kincade. 2003. Unique properties of fetal lymphoid progenitors identified according to RAG1 gene expression. *Immunity* 19: 365–375.

31. Kuwata, N., H. Igarashi, T. Ohmura, S. Aizawa, and N. Sakaguchi. 2001. Cutting edge: absence of expression of RAG1 in peritoneal B-1 cells detected by knocking into RAG1 locus with green fluorescent protein gene. *J. Immunol.* 163: 6355–6359.

32. Lubahn, D. B., J. S. Moyer, T. S. Golding, J. F. Couse, K. S. Korach, and O. Smithies. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc. Natl. Acad. Sci. USA* 90: 11162–11166.

33. Shughrue, P. J., G. R. Askew, T. L. Dellovade, and I. Merchenthaler. 2002. Estrogen-binding sites and their functional capacity in estrogen receptor double knockout mouse brain. *Endocrinology* 143: 1643–1650.

34. Harris, H. A., J. A. Katzenellenbogen, and B. S. Katzenellenbogen. 2002. Characterization of the biological roles of the estrogen receptors, ERα and ERβ, in estrogen target tissues in vivo through the use of an ERα-selective ligand. *Endocrinology* 143: 4172–4177.

35. Harris, H. A., L. M. Albert, Y. Leathurby, M. S. Malamas, R. E. Mewshaw, C. P. Miller, Y. P. Kharode, J. Marzolf, B. S. Komm, R. C. Winneker, et al. 2003. Evaluation of an estrogen receptor-β agonist in animal models of human disease. *Endocrinology* 144: 4241–4249.

36. Kondo, M., I. L. Weissman, and K. Akashi. 1997. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* 91: 661–672.

37. Akashi, K., D. Traver, T. Miyamoto, and I. L. Weissman. 2000. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404: 193–197.

38. Medina, K. L., G. Smithson, and P. W. Kincade. 1993. Suppression of B lymphopoiesis during normal pregnancy. *J. Exp. Med.* 178: 1507–1515.

39. Rijhsinghani, A. G., S. K. Bhatia, L. T. Tygrett, and T. J. Waldschmidt. 1996. Effect of pregnancy on thymic T cell development. *Am. J. Reprod. Immunol.* 35: 523–528.

40. LeBien, T. W. 2000. Fates of human B-cell precursors. *Blood* 96: 9–23.

41. Nishihara, M., Y. Wada, K. Ogami, Y. Ebihara, T. Ishii, K. Tsuji, H. Ueno, S. Asano, S. Nakahata, and T. Maekawa. 1998. A combination of stem cell factor and granulocyte colony-stimulating factor enhances the growth of human progenitor B cells supported by murine stromal cell line MS-5. *Eur. J. Immunol.* 28: 855–864.

42. Oritani, K., K. L. Medina, Y. Tomiyama, J. Ishikawa, Y. Okajima, M. Ogawa, T. Yokota, K. Aoyama, I. Takahashi, P. W. Kincade, and Y. Matsuzawa. 2000.

43. Smithson, G., J. F. Couse, D. B. Lubahn, K. S. Korach, and P. W. Kincade. 1998. The role of estrogen receptors and androgen receptors in sex steroid regulation of B lymphopoiesis. *J. Immunol.* 161: 27–34.

44. Pietrangeli, C. E., S. Hayashi, and P. W. Kincade. 1988. Stromal cell lines which support lymphocyte growth: characterization, sensitivity to radiation and responsiveness to growth factors. *Eur. J. Immunol.* 18: 863–872.

45. Ivanova, N. B., J. T. Dimos, C. Schaniel, J. A. Hackney, K. A. Moore, and I. R. Lemischka. 2002. A stem cell molecular signature. *Science* 298: 601–604.

46. Wong, G. T., B. J. Gavin, and A. P. McMahon. 1994. Differential transformation of mammary epithelial cells by Wnt genes. *Mol. Cell. Biol.* 14: 6278–6286.

47. Scheller, M., J. Huelsken, F. Rosenbauer, M. M. Taketo, W. Birchmeier, D. G. Tenen, and A. Leutz. 2006. Hematopoietic stem cell and multilineage defects generated by constitutive β-catenin activation. *Nat. Immunol.* 7: 1037–1047.

48. Kirstetter, P., K. Anderson, B. T. Porse, S. E. W. Jacobsen, and C. Nerlov. 2006. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nat. Immunol.* 7: 1048–1056.

49. Miller, J. P., and D. Allman. 2003. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. *J. Immunol.* 171: 2326–2330.

50. Montecino-Rodriguez, E., and K. Dorshkind. 2006. Evolving patterns of lymphopoiesis from embryogenesis through senescence. *Immunity* 24: 659–662.

51. Yoshida, T., S. Y. Ng, J. C. Zuniga-Pflucker, and K. Georgopoulos. 2006. Early hematopoietic lineage restrictions directed by Ikaros. *Nat. Immunol.* 7: 382–391.

52. Falkoff, R. 1987. Maternal immunologic changes during pregnancy: a critical appraisal. *Clin. Rev. Allergy* 5: 287–300.

53. Poole, J. A., and H. N. Claman. 2004. Immunology of pregnancy: implications for the mother. *Clin. Rev. Allergy Immunol.* 26: 161–170.

54. Kouro, T., T. Yokota, R. Welner, and P. W. Kincade. 2005. In vitro differentiation and measurement of B cell progenitor activity in culture. In *Current Protocols in Immunology.* J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. eds. John Wiley and Sons, Hoboken, NJ. pp. 2.1–2.12.

55. Ichii, M., K. Oritani, T. Yokota, M. Nishida, I. Takahashi, T. Shirogane, S. Ezoe, N. Saitoh, R. Tanigawa, Y. Kanakura, and Y. Kanakura. 2008. Regulation of human B lymphopoiesis by the transforming growth factor-β superfamily in a newly established coculture system using human mesenchymal stem cells as a supportive microenvironment. *Exp. Hematol.* 36: 587–597.

56. Bosco, N., R. Ceredig, and A. Rolink. 2008. Transient decrease in interleukin-7 availability arrests B lymphopoiesis during pregnancy. *Eur. J. Immunol.* 38: 381–390.

57. Leimeister, C., A. Bach, and M. Gessler. 1998. Developmental expression patterns of mouse sFRP genes encoding members of the secreted frizzled related protein family. *Mech. Dev.* 75: 29–42.

58. Bodine, P. V., W. Zhao, Y. P. Kharode, F. J. Bex, A. J. Lambert, M. B. Goad, T. Gaur, G. S. Stein, J. B. Lian, and B. S. Komm. 2004. The Wnt antagonist secreted frizzled-related protein-1 is a negative regulator of trabecular bone formation in adult mice. *Mol. Endocrinol.* 18: 1222–1237.

59. Zhang, J., C. Niu, L. Ye, H. Huang, X. He, W. G. Tong, J. Ross, J. Haug, T. Johnson, J. Q. Feng, et al. 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 425: 836–841.

60. Calvi, L. M., G. B. Adams, K. W. Weibrecht, J. M. Weber, D. P. Olson, M. C. Knight, R. P. Martin, E. Schipani, P. Divieti, F. R. Bringhurst, et al. 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 425: 841–846.

61. Finch, P. W., X. He, M. J. Kelley, A. Üren, R. P. Schaudies, N. C. Popescu, S. Rudikoff, S. A. Aaronson, H. E. Varmus, and J. S. Rubin. 1997. Purification and molecular cloning of a secreted, frizzled-related antagonist of Wnt action. *Proc. Natl. Acad. Sci. USA* 94: 6770–6775.

62. Üren, A., F. Reichsman, V. Anest, W. G. Taylor, K. Muraiso, D. P. Bottaro, S. Cumberledge, and J. S. Rubin. 2000. Secreted frizzled-related protein-1 binds directly to wingless and is a biphasic modulator of Wnt signaling. *J. Biol. Chem.* 275: 4374–4382.

63. Bafico, A., A. Gazit, T. Pramila, P. W. Finch, A. Yaniv, and S. A. Aaronson. 1999. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *J. Biol. Chem.* 274: 16180–16187.

64. Melkonyan, H. S., W. C. Chang, J. P. Shapiro, M. Mahadevappa, P. A. Fitzpatrick, M. C. Kiefer, L. D. Tomei, and S. R. Umansky. 1997. SARPs: a family of secreted apoptosis-related proteins. *Proc. Natl. Acad. Sci. USA* 94: 13636–13641.

65. Staal, F. J., and H.C. Clevers. 2005. WNT signalling and haematopoiesis: a WNT-WNT situation. *Nat. Rev. Immunol.* 5: 21–30.

66. Cobas, M., A. Wilson, B. Ernst, S. J. Mancini, H. R. MacDonald, R. Kemler, and F. Radtke. 2004. β-catenin is dispensable for hematopoiesis and lymphopoiesis. *J. Exp. Med.* 199: 221–229.

67. Koch, U., A. Wilson, M. Cobas, R. Kemler, H. R. MacDonald, and F. Radtke. 2008. Simultaneous loss of β- and γ-catenin does not perturb hematopoiesis or lymphopoiesis. *Blood* 111: 160–164.

68. Jeannet, G., M. Scheller, L. Scarpellino, S. Duboux, N. Gardiol, J. Back, F. Kuttler, I. Malanchi, W. Birchmeier, A. Leutz, et al. 2007. Long-term, multilineage hematopoiesis occurs in the combined absence of β-catenin and γ-catenin. *Blood* 111: 142–149.

Limitin: an interferon-like cytokine that preferentially influences B-lymphocyte precursors. *Nat. Med.* 6: 659–666.

**Experimental
Hematology**

Regulation of human B lymphopoiesis by the transforming growth factor-β superfamily in a newly established coculture system using human mesenchymal stem cells as a supportive microenvironment

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Objectives. To characterize and evaluate the validity of a novel coculture system for studying human B-lymphocyte developmental biology.

Materials and Methods. We developed a long-term culture system to produce B lymphocytes from human CD34^{+} cells purified from umbilical cord blood using human mesenchymal stem cells (hMSC) as stroma. We evaluated the effects of several low molecular weight inhibitors, recombinant proteins, and neutralizing antibodies (Abs) as potential regulators of B-lymphocyte development.

Results. Our cocultures of 2000 CD34^{+} cells in the presence of stem cell factor and Flt3-ligand produced $1–5 \times 10^5$ CD10^{+} cells after 4 weeks of culture. Surface IgM^{+} immature B cells began to appear after 4 weeks. We evaluated the negative-regulatory effects of the transforming growth factor (TGF)-β superfamily on human B lymphopoiesis, and found that adding an anti–activin A antibody enhanced generation of CD10^{+} cells two- to three-fold. As well, the proportion of CD10^{+} cells in the generated cells increased markedly, indicating that activin A downregulated B lymphopoiesis more efficiently than myelopoiesis. Addition of TGF-β1 suppressed B-lymphocyte production by 20% to 30%, while addition of an anti–bone morphogenetic protein (BMP)-4 antibody or recombinant BMP-4 had no effect. Therefore, the strength of ability to suppress human B lymphopoiesis seemed to be activin A > TGF-β1 > BMP-4. None of these three factors influenced the emergence of IgM^{+} cells.

Conclusions. hMSC coculture supported human B lymphopoiesis. Activin A selectively suppressed B lymphocyte production. © 2008 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

B lymphocytes develop from hematopoietic stem cells within bone marrow (BM), and play an essential role in immune system function. B-lymphocyte production is regulated by an elaborate scheme involving many different soluble or adhesion molecules; failure to control production adequately can promote the development of diseases with quantitative and/or qualitative B-lymphocyte abnormalities

[1]. A variety of murine assay systems are used to evaluate the mechanisms of B-lymphocyte regulation, such as Whitlock-Witte–type long-term BM cultures, cocultures of murine BM cells on stromal cell lines, and colony assays. Studies utilizing these culture systems have elucidated many regulatory mechanisms of B-lymphocyte development in mice. For example, CD44 [2], vascular cell adhesion molecule-1, and very-late activation antigen-4 [3] are adhesion molecules essential for B lymphopoiesis. We have also reported that a novel interferon-ζ/limitin suppressed colony formation of B-lymphocyte progenitors [4,5] and that an adipocyte-specific protein, adiponectin,

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inhibited stroma-dependent B-lymphocyte growth through induction of prostaglandins (PGs) [6]. In addition, members of the TGF superfamily [7], the Wnt family [8], and the Notch family [9] are known to regulate proliferation, differentiation, and survival, depending on the developmental stage of the B lymphocytes. Although early B-lymphocyte development was believed to require direct interactions with supportive stromal layers, in previous work we generated CD45RA^{+}CD19^{+} B lymphocytes from murine Lin[-]c-kit^{high} and Lin[-]c-kit^{low} cells in the presence of stem cell factor (SCF), Flt3-ligand (FL), and interleukin (IL)-7, in the absence of stromal cells [10,11].

In contrast to mice, systems to evaluate human B-lymphocyte developmental biology are not fully established because of a lack of appropriate human stromal materials. Several culture systems for human hematopoietic cells have utilized murine stromal cell lines as supportive microenvironment [12–15]. Although murine stromal cells produce cytokines that could potentially affect human B-lymphocyte development, some of these molecules have no interspecies cross-reactivity [16]. As well, there are some mechanistic differences between murine and human B-lymphocyte development. For example, signaling through the IL-7 receptor (IL-7R) is critical for adult murine B-lymphocyte development [17,18]. However, in humans, disrupting IL-7R does not induce arrest of B lymphopoiesis [19,20]. In addition, human B-lymphocyte progenitor cells cannot expand without stroma [12], whereas murine progenitor cells can [10,11]. Therefore, assay systems more relevant to human biology are needed. A previous study reported the establishment of a serum-free human BM stromal cell culture; however, the investigators isolated cellular components from fetal BM, which is currently unavailable for research purposes [21,22].

In this study, we show that human mesenchymal stem cells (hMSC) can support the commitment and differentiation of human CD34^{+} cells into B lymphocytes. Our cocultures of 2000 human CD34^{+} cells on hMSC in the presence of SCF and FL produced $1-5 \times 10^5$ CD10^{+} B lymphocytes after 4 weeks. Using this coculture system, we determined that members of the transforming growth factor-β (TGF-β) superfamily, activin A and TGF-β1, were negative regulators for early onset of human B lymphopoiesis. The TGF-β superfamily has more than 20 members, including three TGF-βs, two inhibins, three activins, seven bone morphogenetic proteins (BMPs), and nodal [23]. There are two types of receptors for the TGF-β superfamily, type I (activin receptor-like kinase [ALK]-1-7) and type II receptors [24]. Their specific ligand–receptor interactions induce critical effects on a wide range of physiological and pathological processes, such as immune responses, angiogenesis, tumor development, and wound healing [25]. In addition, TGF-βs, activin A, and BMPs have been reported to influence lymphohematopoiesis [7,26,27]. Here, we explore the similarities and/or differences of functions of the TGF-β superfamily members between humans and mice by comparing our results obtained from the human B-lymphocyte coculture with data from several previous reports.

Materials and methods

Origin and isolation of cells
Cord blood (CB) cells were collected from healthy, full-term neonates immediately after delivery by Cesarean section. All participants provided prior informed consent. Mononuclear cells were separated by Ficoll-Paque PLUS (GE Healthcare Bio-Science AB, Uppsala, Sweden) centrifugation. CB CD34^{+} cells were purified using the Direct CD34 Progenitor Cell Isolation Kit (human; Miltenyi Biotec, Auburn, CA, USA). BM-derived hMSC were purchased from Cambrex Bio Science Walkersville (Walkersville, MD, USA) and maintained in MSC Growth Medium (Cambrex Bio Science Walkersville). Human umbilical vein endothelial cells (HUVEC) were purchased from Cascade Biologics (Portland, OR, USA), and maintained in Humedia-EG2 (Kurabo, Osaka, Japan). The murine stromal cell line MS-5, kindly provided by Dr. Mori (Niigata University), was maintained in α-minimum essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS).

Recombinant proteins and reagents
Recombinant human SCF, FL, IL-7, TGF-β1, activin A, and BMP-4 proteins were purchased from R&D Systems (Minneapolis, NY, USA). Granulocyte-colony stimulating factor (G-CSF) was gifted from Kirin Brewery (Tokyo, Japan). N-acetylcysteine was purchased from Sigma (St. Louis, MO, USA), DUP697 from Cayman Chemicals (Ann Arbor, MI, USA), BIO from Calbiochem (Darmstadt, Germany), and SB431542 from TOCRIS Bioscience (Ellisville, MO, USA). Neutralizing antibodies (Abs) against human TGF-β1, activin A, and BMP-4 were purchased from R&D Systems. Follistatin was purchased from Calbiochem.

Cocultures for human B lymphocytes
hMSC were seeded in 12-well tissue culture plates (Iwaki, Tokyo, Japan) 1 or 2 days before coculture. Isolated CB CD34^{+} cells (2000 cells/well) were plated on subconfluent hMSC layers in MSC growth medium in the presence of 10 ng/mL SCF and 5 ng/mL FL. Half of culture medium was replaced with fresh medium containing the same cytokines twice per week. When appropriate, the cultured cells on hMSC were collected and their phenotypes were analyzed with flow cytometry. In some experiments, cultures were performed in medium containing low molecular weight inhibitors or neutralizing Abs, as indicated. In other experiments, HUVEC or MS-5 cells were used as stroma. In cocultures containing HUVEC or MS-5 cells, the culture media were Iscove's modified Eagle's medium (Gibco) supplemented with 20% FCS and 2 mM glutamine or α-minimum essential medium supplemented with 10% FCS, respectively.

Flow cytometry and cell sorting
Flow cytometry analysis was performed with a FACSCalibur (BD Biosciences Immunocytometry Systems, San Jose, CA, USA) using standard multicolor immunofluorescent staining protocols [28]. Murine monoclonal Abs against the following human cell surface molecules were purchased: phycoerythrin (PE)-CD3,

PE-CD10, allophycocyanin (APC)-CD10, PE-CD19, PE-CD20, fluorescein isothiocyanate (FITC)-CD33, APC-CD33, PE-CD34, APC-CD34, FITC-CD38, FITC-CD45, and PE-glycophorin A from BD Biosciences/BD Pharmingen; PC5-CD19 from Beckman Coulter (Marseilles, France), FITC-IgM from Southern Biotechnology Associates (Birmingham, AL, USA). Cultured cells were categorized as myeloid lineage cells (CD33$^+$ and CD10$^-$), B lymphoid lineage cells (CD33$^-$ and CD10$^+$/CD19$^+$), or immature B cell (CD33$^-$, CD10$^+$, CD19$^+$, and IgM$^+$). In some experiments, CD34$^+$CD38$^-$, CD34$^+$CD38$^+$CD10$^-$, and CD34$^+$CD38$^+$CD10$^+$ cells were sorted using a FACS Aria (BD Biosciences Immunocytometry Systems).

Limiting dilution assays
Limiting dilution assays were performed in 96-well plates (Iwaki) preseeded with hMSC. CB CD34$^+$ cells were plated at various concentrations from 1 to 100 cells/well. Each well contained 200 μL MSC Growth Medium with 10 ng/mL SCF and 5 ng/mL FL, with or without 10 μM SB431542. Half of culture medium was replaced with fresh medium containing the same cytokines twice per week. After 28 days of coculture, wells with cell expansion were scored. Individual expanded cells were analyzed by flow cytometry, and the number of culture wells containing CD10$^+$ cells was determined.

Reverse transcription polymerase chain reaction
Total RNA was extracted from CB cells and hMSCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNAs were reverse transcribed and target cDNAs were amplified by polymerase chain reaction using 0.5 U Taq DNA polymerase (Applied Biosystems, Branchburg, NJ, USA; primers sequences are available upon request) [29].

Enzyme-linked immunosorbent assay
TGF-β1 was detected using Immunoassay Kit (Biosource International, Camarillo, CA, USA; sensitivity 15.6 pg/mL). Activin A was detected using DuoSet ELISA Development System (R&D Systems; sensitivity 117.2 pg/mL). Each step of the reactions was performed according to manufacturer's instructions.

Statistical analysis
Student's *t*-test was used to analyze statistically significant differences between data sets. All results are reported as mean values ± standard deviation.

Results

hMSC support B-lymphocyte development from human CB CD34$^+$ cells

The murine stromal cell line MS-5 is used widely to support murine and human lymphohematopoietic cells in culture [12,13]. HUVECs express several adhesion molecules, such as vascular cell adhesion molecule-1 [30], that interact with B lymphocytes. hMSC have the ability to support human hematopoietic stem cells in culture [31], and can enhance engraftment of human hematopoietic stem cell transplantation [32]. Therefore, we compared the ability of these three cell types to support

human B lymphopoiesis in coculture. When purified human CB CD34$^+$ cells were cultured on each different monolayer, CD19$^+$ cells were generated within 4 weeks. As shown in Figure 1, cocultures on hMSCs generated many more CD19$^+$ cells than those on MS-5 or HUVEC. In addition, similar supporting activity for human B lymphopoiesis was observed for two different lots of hMSC (data not shown). Finally, HUVEC started to detach from the culture wells within 3 weeks of culture, while hMSC did not.

Therefore, hMSC appear to better support human B-lymphocyte progenitor cell development than HUVEC or MS-5.

SCF and FL enhance human B lymphopoiesis in coculture

In mice, SCF, FL, and IL-7 are critical for early B-lymphocyte development [10,11]. Therefore, coculture of human CB CD34$^+$ cells on hMSC included various combinations of SCF, FL, and IL-7. Although the addition of SCF, FL, or IL-7 individually to the cocultures enhanced production of B lymphocytes slightly (data not shown), many more B lymphocytes were recovered when these factors were added in combination (Fig. 2A). Anti-CD33 Ab recognizes a 67-kD type I transmembrane glycoprotein expressed mainly on monocytes, granulocytes, and myeloid progenitors, but not on lymphocytes and hematopoietic stem cells. Anti-
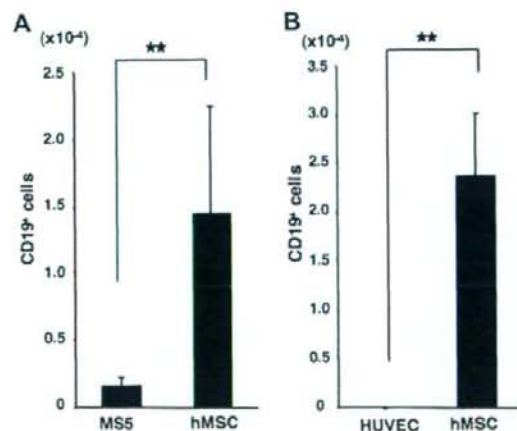
Figure 1. Human mesenchymal stem cells (hMSC) have high supporting activity of human B lymphocytes. Purified cord blood (CB) CD34$^+$ cells (2000 cells/well) were cultured on subconfluent of hMSC (**A, B**), MS-5 (**A**), or human umbilical vein endothelial cells (**B**) in the absence of any cytokines for 4 weeks. Total numbers of the generated cells were calculated, and surface phenotypes of the cells were analyzed with flow cytometry. Data are shown as mean ± standard deviation of the generated CD19$^+$ cell numbers in triplicated samples. Statistically differences from control values are shown with two asterisks ($p < 0.01$). Similar results were obtained in three independent experiments.