

tibody induces cell cycle arrest at the G1-S check point and increases CDK inhibitor p21. Interestingly, the 5F8 mAb, another anti-CD26 mAb recognizing a different epitope than 1F7, is much less potent than 1F7 in inducing growth inhibition, suggesting an epitope-dependent growth inhibitory effect of anti-CD26 mAb. This is consistent with previous studies showing that the CD26 1F7 epitope is involved in signal transduction [39, 53, 210]. Karpas 299 cells injected into severe combined immunodeficiency (SCID) mice and then treated with anti-CD26 monoclonal antibody had significant improvements in overall survival [209]. The anti-CD26 monoclonal antibody 14D10, which recognizes the cell membrane-proximal glycosylated region starting with a 20 amino acid flexible stalk region of CD26, inhibited the renal clear cell carcinoma cell line Caki-2 and human renal cell carcinoma in a mouse xenograft model [178]. This was associated with inhibited Akt, internalized cell surface CD26, G1/S cell cycle arrest, down regulation of CDK2, enhanced CDK inhibitor p27^{kip1} expression, and dephosphorylation of retinoblastoma substrate. A humanized monoclonal anti-CD26 antibody, YSCMA (Y's Therapeutics, Tokyo, Japan), is in pre-clinical development for the treatment of cancer and immune disorders.

8.3 Chemotherapy

Transfection of the Jurkat leukemia T-cell line with CD26 resulted in a greater sensitivity to doxorubicin and etoposide compared to untransfected cells [16, 211-213]. Jurkat cells transfected with a nonfunctional DPPIV CD26 catalytic site mutant (Ser630Ala) did not show increased doxorubicin and etoposide sensitivity. Suggesting DPPIV activity is not needed for chemo-sensitization. A CD26 transfectant with a mutation at the ADA binding site retains DPPIV activity and had a higher level of doxorubicin sensitivity. Surface CD26 expression and DPPIV activity are associated with increased doxorubicin sensitivity and cell cycle arrest in Jurkat cells. Also, there are differences in hyperphosphorylation and inhibition of p34^{cdc2} kinase activity, phosphorylation of cdc25C, and alteration in cyclin B1 expression associated with doxorubicin sensitivity in Jurkat cell lines [211]. Therefore, inhibition of CD26 increases cell survival, while increased CD26 expression is associated with decreased drug resistance. The mechanism of this decreased resistance appears to be by CD26 mediated enhanced expression of topoisomerase II α – the target for both doxorubicin and etoposide. This association between CD26 and topoisomerase II α may explain the aggressive behavior of CD26+ T-cell malignancies. Additionally, the increased sensitivity to doxorubicin and etoposide in CD26 expressing tumors may be important in T-cell hematologic malignancies as well as other cancers.

Surface expression of CD26 increases topoisomerase II α level in the B-cell line Jiyoye and increases doxorubicin sensitivity. This was demonstrated by using CD26 transfection constructs in the Jiyoye B-cell lymphoma cell line as well as by target specific siRNA inhibition of CD26 in the Karpas-299 T-cell leukemia cell line. Therefore, CD26 has effects on topoisomerase II α and doxorubicin sensitivity in both B-cell and T-cell lines. Increased CD26/DPPIV levels are associated with increased phosphorylation of p38 and its upstream regulators – mitogen-activated protein kinase

(MAPKK) 3/6 and apoptosis signal-regulating kinase 1 (ASK1). Therefore, the p38 signaling pathway may be involved in the regulation of topoisomerase II α expression. Doxorubicin treated SCID mice had increased survival in those injected with wild type CD26 compared to vector or CD26 catalytic site mutant (Ser630Ala) injected mice. CD26/DPPIV levels may be useful predictive markers for doxorubicin treatment of cancer.

CD26 level is also associated with etoposide resistance. CD26 mediated changes include hyperphosphorylation of p34^{cdc2}, variation in cdc25C level and phosphorylation, and changes in cyclin B1 level. CD26/DPPIV cleaves substrates resulting in altered function [110, 214]. Therefore, CD26-associated drug sensitivity could be mediated by serum-derived factors. The increased doxorubicin and etoposide sensitivity of CD26+ transfected Jurkat cells was independent of serum, which suggests an effect of CD26 on cell-mediated processes, such as signal transduction, rather than serum-derived factors [212].

Recent studies suggest that topoisomerase II α level is a prognostic factor in breast cancer that is independent of stage, Her-2/neu status, and histological grading [151]. Furthermore, anthracycline treatment did not reverse the negative prognostic effect of topoisomerase II α expression. Others have found in retrospective studies that topoisomerase II α overexpression confers a higher probability of response to doxorubicin [152]. CD26 is associated with increased topoisomerase II α levels and tumor sensitivity to the topoisomerase II inhibitors doxorubicin and etoposide; therefore CD26 may be an important marker in breast cancer. The clinical significance of CD26 in breast cancer is an area for future research.

Therefore, CD26 has an important role in a number of clinical conditions including solid and hematologic malignancies. CD26 may have diagnostic significance (absence in SS/MF) as well as providing prognostic information (colon cancer serum levels of CD26 and disease free survival) and predictive power (doxorubicin and etoposide sensitivity). The implications of CD26/DPPIV as a therapeutic target will be better understood as clinical trials with CD26 inhibitors open and accrue patients.

CONCLUSION

As discussed, CD26 may be exploitable for diagnosis, prognosis, and predictive ability. Additional roles in autoimmune and other diseases are currently being tested in the laboratory and in early human clinical trials. Given the involvement of CD26 in multiple physiologic functions as well as in tumor biology, the development of therapeutic modalities targeting this fascinating molecule may indeed prove to be a useful strategy in the treatment of selected human diseases.

REFERECES

- [1] Hopsu-Havu, V. K.; Glenner, G. G. *Histochemie*, 1966, 7(3), 197.
- [2] Tanaka, T.; Camerini, D.; Seed, B.; Torimoto, Y.; Dang, N. H.; Kameoka, J.; Dahlberg, H. N.; Schlossman, S. F.; Morimoto, C. J. *Immunol.*, 1992, 149(2), 481.
- [3] Morrison, M. E.; Vijayasradhi, S.; Engelstein, D.; Albino, A. P.; Houghton, A. N. J. *Exp. Med.*, 1993, 177(4), 1135.

- [4] Fleischer, B., *Dipeptidyl peptidase IV (CD26) in metabolism and the immune response*. ed.; Springer: New York, 1995; 'Vol.' p 202.
- [5] De Meester, I.; Korom, S.; Van Damme, J.; Scharpe, S. *Immunol. Today*, 1999, 20(8), 367.
- [6] Kahne, T.; Lendeckel, U.; Wrenger, S.; Neubert, K.; Ansorge, S.; Reinhold, D. *Int. J. Mol. Med.*, 1999, 4(1), 3.
- [7] Hildebrandt, M.; Reutter, W.; Arck, P.; Rose, M.; Klapp, B. F. *Clin. Sci. (Lond)*, 2000, 99(2), 93.
- [8] Gorrell, M. D.; Gysbers, V.; McCaughan, G. W. *Scand. J. Immunol.*, 2001, 54(3), 249.
- [9] Langner, J.; Ansorge, S., *Cellular peptidases in immune functions and diseases 2*. ed.; Kluwer Acad./Plenum Publ.: New York, 2000; 'Vol.' p 522.
- [10] Langner, J.; Ansorge, S., *Ectopeptidases CD13/aminopeptidase N and CD26/dipeptidyl peptidase IV in Medicine and Biology*. ed.; Kluwer Acad./Plenum Publ.: New York, 2002; 'Vol.' p 316.
- [11] Boonacker, E.; Van Noorden, C. J. *Eur. J. Cell Biol.*, 2003, 82(2), 53.
- [12] Verstovsek, S.; Cabanillas, F.; Dang, N. H. *Oncology (Williston Park)*, 2000, 14(6 Suppl 2), 17.
- [13] Dang, N. H.; Morimoto, C. *Histol. Histopathol.*, 2002, 17(4), 1213.
- [14] Pro, B.; Dang, N. H. *Histol. Histopathol.*, 2004, 19(4), 1345.
- [15] Aytac, U.; Dang, N. H. *Curr. Drug Targets Immune Endocr. Metabol. Disord.*, 2004, 4(1), 11.
- [16] Sato, K.; Dang, N. H. *Int. J. Oncol.*, 2003, 22(3), 481-97.
- [17] Hildebrandt, M.; Klapp, B.; Hoffmann, T.; Demuth, H. U., 'Ed.' Eds. Kluwer Academic/Plenum Publishers: New York, 2003; 'Vol.' p pp.
- [18] Abbott, C. A.; Yu, D. M.; Woollatt, E.; Sutherland, G. R.; McCaughan, G. W.; Gorrell, M. D. *Eur. J. Biochem.*, 2000, 267(20), 6140.
- [19] Abbott, C. A.; McCaughan, G. W.; Gorrell, M. D. *FEBS Lett.*, 1999, 458(3), 278.
- [20] Abbott, C. A.; Baker, E.; Sutherland, G. R.; McCaughan, G. W. *Immunogenetics*, 1994, 40(5), 331.
- [21] Bohm, S. K.; Gum, J. R., Jr.; Erickson, R. H.; Hicks, J. W.; Kim, Y. S. *Biochem. J.*, 1995, 311 (Pt 3), 835.
- [22] Bauvois, B.; Djavaheri-Mergny, M.; Rouillard, D.; Dumont, J.; Wietzerbin, J. *Oncogene*, 2000, 19(2), 265.
- [23] Erickson, R. H.; Gum, J. R.; Lotterman, C. D.; Hicks, J. W.; Lai, R. S.; Kim, Y. S. *Biochem. J.*, 1999, 338 (Pt 1), 91.
- [24] Misumi, Y.; Hayashi, Y.; Arakawa, F.; Ikehara, Y. *Biochim. Biophys. Acta*, 1992, 1131(3), 333.
- [25] Chihara, C. J.; Song, C.; LaMonte, G.; Fetalvero, K.; Hinchman, K.; Phan, H.; Robinson, K.; Schneider, G. P. *J. Insect Sci.*, 2005, 5(26), 1.
- [26] Engel, M.; Hoffmann, T.; Wagner, L.; Wermann, M.; Heiser, U.; Kiefersauer, R.; Huber, R.; Bode, W.; Demuth, H.-U.; Brandstetter, H. *PNAS*, 2003, 100(9), 5063.
- [27] Rasmussen, H. B.; Branner, S.; Wiberg, F. C.; Wagtmann, N. *Nat. Struct. Mol. Biol.*, 2003, 10(1), 19.
- [28] Yaron, A.; Naider, F. *Crit. Rev. Biochem. Mol. Biol.*, 1993, 28(1), 31.
- [29] Fleischer, B., 'Ed.' Eds. Springer: New York, 1995; 'Vol.' p pp 1.
- [30] David, F.; Bernard, A. M.; Pierres, M.; Marguet, D. *J. Biol. Chem.*, 1993, 268(23), 17247.
- [31] Hooper, J. D.; Clements, J. A.; Quigley, J. P.; Antalis, T. M. *J. Biol. Chem.*, 2001, 276(2), 857.
- [32] Fulop, V.; Bocskei, Z.; Polgar, L. *Cell*, 1998, 94(2), 161.
- [33] Carter, P.; Wells, J. A. *Science*, 1987, 237(4813), 394-9.
- [34] Vahl, T. P.; Paty, B. W.; Fuller, B. D.; Prigeon, R. L.; D'Alessio, D. A. *J. Clin. Endocrinol. Metab.*, 2003, 88(4), 1772.
- [35] Stange, T.; Kettmann, U.; Holzhausen, H. J. *Eur. J. Histochem.*, 2000, 44(2), 157.
- [36] Carbone, A.; Gloghini, A.; Zagonel, V.; Aldinucci, D.; Gattei, V.; Degan, M.; Improta, S.; Sorio, R.; Monfardini, S.; Pinto, A. *Blood*, 1995, 86(12), 4617.
- [37] Carbone, A.; Cozzi, M.; Gloghini, A.; Pinto, A. *Hum. Pathol.*, 1994, 25(12), 1360.
- [38] Kahne, T.; Kroning, H.; Thiel, U.; Ulmer, A. J.; Flad, H. D.; Ansorge, S. *Cell Immunol.*, 1996, 170(1), 63.
- [39] Torimoto, Y.; Dang, N. H.; Tanaka, T.; Prado, C.; Schlossman, S. F.; Morimoto, C. *Mol. Immunol.*, 1992, 29(2), 183.
- [40] Smith, R. E.; Talhouk, J. W.; Brown, E. E.; Edgar, S. E. *AIDS Res. Hum. Retrovir.*, 1998, 14(10), 851.
- [41] Schmauser, B.; Kilian, C.; Reutter, W.; Tauber, R. *Glycobiology*, 1999, 9(12), 1295.
- [42] Mavropoulos, J. C.; Cuchacovich, M.; Llanos, C.; Aguilon, J. C.; Gatica, H.; Pizzo, S. V.; Gonzalez-Gronow, M. *J. Rheumatol.*, 2005, 32(11), 2116.
- [43] Slimane, T. A.; Lenoir, C.; Sapin, C.; Maurice, M.; Trugnan, G. *Exp. Cell Res.*, 2000, 258(1), 184.
- [44] Tirupathi, C.; Miyamoto, Y.; Ganapathy, V.; Leibach, F. H. *Am. J. Physiol.*, 1993, 265(1 Pt 1), G81.
- [45] Kraut, J. *Annu. Rev. Biochem.*, 1977, 46, 331.
- [46] Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.*, 1968, 32(5), 898.
- [47] Lambeir, A. M.; Proost, P.; Durinx, C.; Bal, G.; Senten, K.; Augustyns, K.; Scharpe, S.; Van Damme, J.; De Meester, I. *J. Biol. Chem.*, 2001, 276(32), 29839.
- [48] Hoffmann, T.; Faust, J.; Neubert, K.; Ansorge, S. *FEBS Lett.*, 1993, 336(1), 61.
- [49] Marguet, D.; Baggio, L.; Kobayashi, T.; Bernard, A. M.; Pierres, M.; Nielsen, P. F.; Ribet, U.; Watanabe, T.; Drucker, D. J.; Wagtmann, N. *Proc. Natl. Acad. Sci. USA*, 2000, 97(12), 6874.
- [50] Dong, R. P.; Tachibana, K.; Hegen, M.; Munakata, Y.; Cho, D.; Schlossman, S. F.; Morimoto, C. *J. Immunol.*, 1997, 159(12), 6070.
- [51] Abbott, C. A.; McCaughan, G. W.; Levy, M. T.; Church, W. B.; Gorrell, M. D. *Eur. J. Biochem.*, 1999, 266(3), 798.
- [52] Cheng, H. C.; Abdel-Ghany, M.; Zhang, S.; Pauli, B. U. *Clin. Exp. Metastasis*, 1999, 17(7), 609-15.
- [53] Morimoto, C.; Torimoto, Y.; Levinson, G.; Rudd, C. E.; Schrieber, M.; Dang, N. H.; Letvin, N. L.; Schlossman, S. F. *J. Immunol.*, 1989, 143(11), 3430.
- [54] Dang, N. H.; Hafner, D. A.; Schlossman, S. F.; Breitmeyer, J. B. *Cell Immunol.*, 1990, 125(1), 42.
- [55] Dang, N. H.; Torimoto, Y.; Deusch, K.; Schlossman, S. F.; Morimoto, C. *J. Immunol.*, 1990, 144(11), 4092.
- [56] Dang, N. H.; Torimoto, Y.; Sugita, K.; Daley, J. F.; Schow, P.; Prado, C.; Schlossman, S. F.; Morimoto, C. *J. Immunol.*, 1990, 145(12), 3963.
- [57] Hegen, M.; Mittrucker, H. W.; Hug, R.; Demuth, H. U.; Neubert, K.; Barth, A.; Fleischer, B. *Immunobiology*, 1993, 189(5), 483.
- [58] von Bonin, A.; Huhn, J.; Fleischer, B. *Immunol. Rev.*, 1998, 161, 43.
- [59] Boonacker, E. P.; Wierenga, E. A.; Smits, H. H.; Van Noorden, C. J. *J. Histochem. Cytochem.*, 2002, 50(9), 1169.
- [60] Tanaka, T.; Kameoka, J.; Yaron, A.; Schlossman, S. F.; Morimoto, C. *Proc. Natl. Acad. Sci. USA*, 1993, 90(10), 4586.
- [61] Steeg, C.; Hartwig, U.; Fleischer, B. *Cell Immunol.*, 1995, 164(2), 311.
- [62] Huhn, J.; Ehrlich, S.; Fleischer, B.; von Bonin, A. *Immunol. Lett.*, 2000, 72(2), 127.
- [63] Dang, N. H.; Torimoto, Y.; Schlossman, S. F.; Morimoto, C. *J. Exp. Med.*, 1990, 172(2), 649.
- [64] Dang, N. H.; Torimoto, Y.; Shimamura, K.; Tanaka, T.; Daley, J. F.; Schlossman, S. F.; Morimoto, C. *J. Immunol.*, 1991, 147(9), 2825.
- [65] Bermpohl, F.; Loster, K.; Reutter, W.; Baum, O. *FEBS Lett.*, 1998, 428(3), 152.
- [66] Savino, W.; Villa-Verde, D. M.; Lannes-Vieira, J. *Immunol. Today*, 1993, 14(4), 158.
- [67] Vissinga, C. S.; Dirven, C. J.; Steinmeyer, F. A.; Benner, R.; Boersma, W. J. *Cell Immunol.*, 1987, 108(2), 323.
- [68] Ohnuma, K.; Munakata, Y.; Ishii, T.; Iwata, S.; Kobayashi, S.; Hosono, O.; Kawasaki, H.; Dang, N. H.; Morimoto, C. *J. Immunol.*, 2001, 167(12), 6745.
- [69] Tanaka, T.; Duke-Cohan, J. S.; Kameoka, J.; Yaron, A.; Lee, I.; Schlossman, S. F.; Morimoto, C. *Proc. Natl. Acad. Sci. USA*, 1994, 91(8), 3082.
- [70] Ishii, T.; Ohnuma, K.; Murakami, A.; Takasawa, N.; Kobayashi, S.; Dang, N. H.; Schlossman, S. F.; Morimoto, C. *Proc. Natl. Acad. Sci. USA*, 2001, 98(21), 12138.
- [71] Torimoto, Y.; Dang, N. H.; Vivier, E.; Tanaka, T.; Schlossman, S. F.; Morimoto, C. *J. Immunol.*, 1991, 147(8), 2514.
- [72] Montixi, C.; Langlet, C.; Bernard, A. M.; Thimonier, J.; Dubois, C.; Wurbel, M. A.; Chauvin, J. P.; Pierres, M.; He, H. T. *EMBO J.*, 1998, 17(18), 5334.
- [73] Xavier, R.; Brennan, T.; Li, Q.; McCormack, C.; Seed, B. *Immunol.*, 1998, 8(6), 723.

- [74] Horejsi, V.; Drbal, K.; Cebecauer, M.; Cerny, J.; Brdicka, T.; Angelisova, P.; Stockinger, H. *Immunol. Today*, 1999, 20(8), 356.
- [75] Janes, P. W.; Ley, S. C.; Magee, A. I. *J. Cell Biol.*, 1999, 147(2), 447.
- [76] Yashiro-Ohtani, Y.; Zhou, X. Y.; Toyo-Oka, K.; Tai, X. G.; Park, C. S.; Hamaoka, T.; Abe, R.; Miyake, K.; Fujiwara, H. *J. Immunol.*, 2000, 164(3), 1251.
- [77] Proost, P.; Schutyser, E.; Menten, P.; Struyf, S.; Wuyts, A.; Opdenakker, G.; Dethoux, M.; Parmentier, M.; Durinx, C.; Lambeir, A. M.; Neyts, J.; Liekens, S.; Maudgal, P. C.; Billiau, A.; Van Damme, J. *Blood*, 2001, 98(13), 3554.
- [78] Eguchi, K.; Ueki, Y.; Shimomura, C.; Otsubo, T.; Nakao, H.; Migita, K.; Kawakami, A.; Matsunaga, M.; Tezuka, H.; Ishikawa, N.; *et al.* *J. Immunol.*, 1989, 142(12), 4233.
- [79] Gerli, R.; Muscat, C.; Bertotto, A.; Bistoni, O.; Agea, E.; Tognellini, R.; Fiorucci, G.; Cesarotti, M.; Bombardieri, S. *Clin. Immunol. Immunopathol.*, 1996, 80(1), 31.
- [80] Hafler, D. A.; Fox, D. A.; Manning, M. E.; Schlossman, S. F.; Reinherz, E. L.; Weiner, H. L. *N. Engl. J. Med.*, 1985, 312(22), 1405.
- [81] Mizokami, A.; Eguchi, K.; Kawakami, A.; Ida, H.; Kawabe, Y.; Tsukada, T.; Aoyagi, T.; Maeda, K.; Morimoto, C.; Nagataki, S. *J. Rheumatol.*, 1996, 23(12), 2022.
- [82] Muscat, C.; Bertotto, A.; Agea, E.; Bistoni, O.; Ercolani, R.; Tognellini, R.; Spinuzzi, F.; Cesarotti, M.; Gerli, R. *Clin. Exp. Immunol.*, 1994, 98(2), 252.
- [83] Masuyama, J.; Berman, J. S.; Cruikshank, W. W.; Morimoto, C.; Center, D. M. *J. Immunol.*, 1992, 148(5), 1367.
- [84] Shingu, K.; Helfritz, A.; Zielinska-Skowronek, M.; Meyer-Olson, D.; Jacobs, R.; Schmidt, R. E.; Mentlein, R.; Pabst, R.; von Horsten, S. *Cancer Immunol. Immunother.*, 2003, 52(9), 546.
- [85] Ohnuma, K.; Yamochi, T.; Uchiyama, M.; Nishibashi, K.; Yoshikawa, N.; Shimizu, N.; Iwata, S.; Tanaka, H.; Dang, N. H.; Morimoto, C. *Proc. Natl. Acad. Sci. USA*, 2004, 101(39), 14186.
- [86] Kameoka, J.; Tanaka, T.; Nojima, Y.; Schlossman, S. F.; Morimoto, C. *Science*, 1993, 261(5120), 466.
- [87] Dong, R. P.; Kameoka, J.; Hegen, M.; Tanaka, T.; Xu, Y.; Schlossman, S. F.; Morimoto, C. *J. Immunol.*, 1996, 156(4), 1349.
- [88] Tsuboi, I.; Sagawa, K.; Shichijo, S.; Yokoyama, M. M.; Ou, D. W.; Wiederhold, M. D. *Clin. Diagn. Lab. Immunol.*, 1995, 2(5), 626.
- [89] Martin, M.; Huguet, J.; Centelles, J. J.; Franco, R. *J. Immunol.*, 1995, 155(10), 4630.
- [90] Franco, R.; Valenzuela, A.; Lluís, C.; Blanco, J. *Immunol. Rev.*, 1998, 161, 27.
- [91] De Meester, I.; Vanham, G.; Kestens, L.; Vanhoof, G.; Bosmans, E.; Gigase, P.; Scharpe, S. *Eur. J. Immunol.*, 1994, 24(3), 566.
- [92] Valenzuela, A.; Blanco, J.; Callebaut, C.; Jacotot, E.; Lluís, C.; Hovanessian, A. G.; Franco, R. *J. Immunol.*, 1997, 158(8), 3721.
- [93] Richard, E.; Arredondo-Vega, F. X.; Santisteban, I.; Kelly, S. J.; Patel, D. D.; Hershfield, M. S. *J. Exp. Med.*, 2000, 192(9), 1223.
- [94] Morimoto, C.; Schlossman, S. F. *Immunol. Rev.*, 1998, 161, 55.
- [95] Dang, N. H.; Hagemeister, F. B.; Duvic, M.; Romaguera, J. E.; Younes, A.; Jones, D.; Samuels, B.; Fayad, L. E.; Pro, B.; Samaniego, F.; Sarris, A.; Goy, A.; McLaughlin, P.; Tong, A. T.; Walker, P. L.; Tiongson, L. P.; Smith, T. L.; Huh, Y. O.; Morimoto, C.; Rodriguez, M. A. *Oncol. Rep.*, 2003, 10(5), 1513.
- [96] Richard, E.; Alam, S. M.; Arredondo-Vega, F. X.; Patel, D. D.; Hershfield, M. S. *J. Biol. Chem.*, 2002, 277(22), 19720-6.
- [97] Huhn, J.; Olek, S.; Fleischer, B.; von Bonin, A. *Cell Immunol.*, 1999, 192(1), 33.
- [98] Sato, T.; Yamochi, T.; Yamochi, T.; Aytac, U.; Ohnuma, K.; McKee, K. S.; Morimoto, C.; Dang, N. H. *Cancer Res.*, 2005, 65(15), 6950.
- [99] Ikushima, H.; Munakata, Y.; Ishii, T.; Iwata, S.; Terashima, M.; Tanaka, H.; Schlossman, S. F.; Morimoto, C. *Proc. Natl. Acad. Sci. USA*, 2000, 97(15), 8439.
- [100] Ikushima, H.; Munakata, Y.; Iwata, S.; Ohnuma, K.; Kobayashi, S.; Dang, N. H.; Morimoto, C. *Cell Immunol.*, 2002, 215(1), 106.
- [101] Morimoto, C.; Lord, C. I.; Zhang, C.; Duke-Cohan, J. S.; Letvin, N. L.; Schlossman, S. F. *Proc. Natl. Acad. Sci. USA*, 1994, 91(21), 9960.
- [102] Gaetaniello, L.; Fiore, M.; de Filippo, S.; Pozzi, N.; Tamasi, S.; Pignata, C. *Hepatology*, 1998, 27(4), 934.
- [103] Bauvois, B. *Biochem. J.*, 1988, 252(3), 723.
- [104] Loster, K.; Zeilinger, K.; Schuppan, D.; Reutter, W. *Biochem. Biophys. Res. Commun.*, 1995, 217(1), 341.
- [105] Piazza, G. A.; Callanan, H. M.; Mowery, J.; Hixson, D. C. *Biochem. J.*, 1989, 262(1), 327.
- [106] Cheng, H. C.; Abdel-Ghany, M.; Elble, R. C.; Pauli, B. U. *J. Biol. Chem.*, 1998, 273(37), 24207.
- [107] Hanski, C.; Huhle, T.; Gossrau, R.; Reutter, W. *Exp. Cell Res.*, 1988, 178(1), 64.
- [108] Cheng, H. C.; Abdel-Ghany, M.; Pauli, B. U. *J. Biol. Chem.*, 2003, 278(27), 24600.
- [109] Herrera, C.; Morimoto, C.; Blanco, J.; Mallol, J.; Arenzana, F.; Lluís, C.; Franco, R. *J. Biol. Chem.*, 2001, 276(22), 19532.
- [110] Oravec, T.; Pall, M.; Roderiquez, G.; Gorrell, M. D.; Ditto, M.; Nguyen, N. Y.; Boykins, R.; Unsworth, E.; Norcross, M. A. *J. Exp. Med.*, 1997, 186(11), 1865.
- [111] Shioda, T.; Kato, H.; Ohnishi, Y.; Tashiro, K.; Ikegawa, M.; Nakayama, E. E.; Hu, H.; Kato, A.; Sakai, Y.; Liu, H.; Honjo, T.; Nomoto, A.; Iwamoto, A.; Morimoto, C.; Nagai, Y. *Proc. Natl. Acad. Sci. USA*, 1998, 95(11), 6331.
- [112] Ohtsuki, T.; Hosono, O.; Kobayashi, H.; Munakata, Y.; Souta, A.; Shioda, T.; Morimoto, C. *FEBS Lett.*, 1998, 431(2), 236.
- [113] Proost, P.; Struyf, S.; Schols, D.; Durinx, C.; Wuyts, A.; Lenaerts, J. P.; De Clercq, E.; De Meester, I.; Van Damme, J. *FEBS Lett.*, 1998, 432(1-2), 73.
- [114] Narducci, M. G.; Scala, E.; Bresin, A.; Caprini, E.; Picchio, M. C.; Remotti, D.; Ragone, G.; Nasorri, F.; Frontani, M.; Arcelli, D.; Volinia, S.; Lombardo, G. A.; Baliva, G.; Napolitano, M.; Russo, G. *Blood*, 2006, 107(3), 1108.
- [115] Fox, D. A.; Hussey, R. E.; Fitzgerald, K. A.; Acuto, O.; Poole, C.; Palley, L.; Daley, J. F.; Schlossman, S. F.; Reinherz, E. L. *J. Immunol.*, 1984, 133(3), 1250.
- [116] Natarajan, C.; Bright, J. J. *J. Immunol.*, 2002, 168(12), 6506.
- [117] Hafler, D. A.; Chofflon, M.; Benjamin, D.; Dang, N. H.; Breitmeyer, J. *J. Immunol.*, 1989, 142(8), 2590.
- [118] Callebaut, C.; Krust, B.; Jacotot, E.; Hovanessian, A. G. *Science*, 1993, 262(5142), 2045.
- [119] Lane, H. C.; Depper, J. M.; Greene, W. C.; Whalen, G.; Waldmann, T. A.; Fauci, A. S. *N. Engl. J. Med.*, 1985, 313(2), 79.
- [120] van Noesel, C. J.; Gruters, R. A.; Terpstra, F. G.; Schellekens, P. T.; van Lier, R. A.; Miedema, F. J. *Clin. Invest.*, 1990, 86(1), 293.
- [121] Schnittman, S. M.; Lane, H. C.; Greenhouse, J.; Justement, J. S.; Baseler, M.; Fauci, A. S. *Proc. Natl. Acad. Sci. USA*, 1990, 87(16), 6058.
- [122] Blazquez, M. V.; Madueno, J. A.; Gonzalez, R.; Jurado, R.; Bachovchin, W. W.; Pena, J.; Munoz, E. *J. Immunol.*, 1992, 149(9), 3073.
- [123] Schmitz, T.; Underwood, R.; Khirroya, R.; Bachovchin, W. W.; Huber, B. T. *J. Clin. Invest.*, 1996, 97(6), 1545.
- [124] Proost, P.; Struyf, S.; Wuyts, A.; Menten, P.; De Meester, I.; Lambeir, A. M.; Scharpe, S.; Schols, D.; De Clercq, E.; Van Damme, J. *Eur. Cytokine Netw.*, 1998, 9(3 Suppl), 73.
- [125] Proost, P.; Menten, P.; Struyf, S.; Schutyser, E.; De Meester, I.; Van Damme, J. *Blood*, 2000, 96(5), 1674.
- [126] Struyf, S.; Menten, P.; Lenaerts, J. P.; Put, W.; D'Haese, A.; De Clercq, E.; Schols, D.; Proost, P.; Van Damme, J. *Eur. J. Immunol.*, 2001, 31(7), 2170.
- [127] Weihofen, W. A.; Liu, J.; Reutter, W.; Saenger, W.; Fan, H. *J. Biol. Chem.*, 2005, 280(15), 14911.
- [128] Cordero, O. J.; Salgado, F. J.; Mera-Varela, A.; Nogueira, M. *Rheumatol. Int.*, 2001, 21(2), 69.
- [129] Ohnuma, K.; Inoue, H.; Uchiyama, M.; Yamochi, T.; Hosono, O.; Dang, N. H.; Morimoto, C. *Mod. Rheumatol.*, 2006, 16(1), 3.
- [130] Tanaka, S.; Murakami, T.; Horikawa, H.; Sugiura, M.; Kawashima, K.; Sugita, T. *Int. J. Immunopharmacol.*, 1997, 19(1), 15.
- [131] Maher, K. J.; Klimas, N. G.; Fletcher, M. A. *Clin. Exp. Immunol.*, 2005, 142(3), 505.
- [132] Mentlein, R.; Gallwitz, B.; Schmidt, W. E. *Eur. J. Biochem.*, 1993, 214(3), 829.
- [133] Kieffer, T. J.; McIntosh, C. H.; Pederson, R. A. *Endocrinology*, 1995, 136(8), 3585.
- [134] Deacon, C. F.; Nauck, M. A.; Toft-Nielsen, M.; Pridal, L.; Willms, B.; Holst, J. J. *Diabetes*, 1995, 44(9), 1126.
- [135] Pauly, R. P.; Rosche, F.; Wermann, M.; McIntosh, C. H.; Pederson, R. A.; Demuth, H. U. *J. Biol. Chem.*, 1996, 271(38), 23222.
- [136] Knudsen, L. B.; Pridal, L. *Eur. J. Pharmacol.*, 1996, 318(2-3), 429.

- [137] Wettergren, A.; Wojdemann, M.; Holst, J. J. *Peptides*, 1998, 19(5), 877.
- [138] Deacon, C. F.; Nauck, M. A.; Meier, J.; Hucking, K.; Holst, J. J. *J. Clin. Endocrinol. Metab.*, 2000, 85(10), 3575.
- [139] Holst, J. J.; Deacon, C. F. *Diabetes*, 1998, 47(11), 1663.
- [140] Salehi, M.; D'Alessio, D. A. *Cleve. Clin. J. Med.*, 2006, 73(4), 382.
- [141] Pederson, R. A.; White, H. A.; Schlenzig, D.; Pauly, R. P.; McIntosh, C. H.; Demuth, H. U. *Diabetes*, 1998, 47(8), 1253.
- [142] Balkan, B.; Kwasnik, L.; Miserendino, R.; Holst, J. J.; Li, X. *Diabetologia*, 1999, 42(11), 1324.
- [143] Ahren, B.; Simonsson, E.; Larsson, H.; Landin-Olsson, M.; Torgeirsson, H.; Jansson, P. A.; Sandqvist, M.; Bavenholm, P.; Efendic, S.; Eriksson, J. W.; Dickinson, S.; Holmes, D. *Diabetes Care*, 2002, 25(5), 869.
- [144] Reimer, M. K.; Holst, J. J.; Ahren, B. *Eur. J. Endocrinol.*, 2002, 146(5), 717.
- [145] Nagakura, T.; Yasuda, N.; Yamazaki, K.; Ikuta, H.; Yoshikawa, S.; Asano, O.; Tanaka, I. *Biochem. Biophys. Res. Commun.*, 2001, 284(2), 501.
- [146] Khin, E. E.; Kikkawa, F.; Ino, K.; Kajiyama, H.; Suzuki, T.; Shibata, K.; Tamakoshi, K.; Nagasaka, T.; Mizutani, S. *Am. J. Obstet. Gynecol.*, 2003, 188(3), 670.
- [147] Nishikawa, M.; Itakura, A.; Ito, M.; Takeuchi, M.; Sato, Y.; Kajiyama, H.; Mizutani, S.; Kikkawa, F. *Horm. Metab. Res.*, 2005, 37(7), 408.
- [148] Hildebrandt, M.; Arck, P. C.; Kruber, S.; Demuth, H. U.; Reutter, W.; Klapp, B. F. *Scand. J. Immunol.*, 2001, 53(5), 449.
- [149] Gonzalez-Gronow, M.; Grenett, H. E.; Weber, M. R.; Gawdi, G.; Pizzo, S. V. *Biochem. J.*, 2001, 355(Pt 2), 397.
- [150] Abdel-Ghany, M.; Cheng, H.; Levine, R. A.; Pauli, B. U. *Invasion Metastasis*, 1998, 18(1), 35.
- [151] Fritz, P.; Cabrera, C. M.; Dippon, J.; Gerteis, A.; Simon, W.; Aulitzky, W. E.; van der Kuip, H. *Breast Cancer Res.*, 2005, 7(3), R374.
- [152] Durbecq, V.; Paesmans, M.; Cardoso, F.; Desmedt, C.; Di Leo, A.; Chan, S.; Friedrichs, K.; Pinter, T.; Van Belle, S.; Murray, E.; Boddrog, I.; Walpole, E.; Lesperance, B.; Korec, S.; Crown, J.; Simmonds, P.; Perren, T. J.; Leroy, J. Y.; Rouas, G.; Sotiriou, C.; Piccart, M.; Larsimont, D. *Mol. Cancer Ther.*, 2004, 3(10), 1207.
- [153] Cordero, O. J.; Ayude, D.; Nogueira, M.; Rodriguez-Bercoval, F. J.; de la Cadena, M. P. *Br. J. Cancer*, 2000, 83(9), 1139.
- [154] Adams, S.; Miller, G. T.; Jesson, M. I.; Watanabe, T.; Jones, B.; Wallner, B. P. *Cancer Res.*, 2004, 64(15), 5471.
- [155] Mizokami, Y.; Kajiyama, H.; Shibata, K.; Ino, K.; Kikkawa, F.; Mizutani, S. *Int. J. Cancer*, 2004, 110(5), 652.
- [156] Sedo, A.; Malik, R.; Krepela, E. *Biol. Chem.*, 1998, 379(1), 39.
- [157] Sedo, A.; Busek, P.; Scholzova, E.; Malik, R.; Vlasicova, K.; Janackova, S.; Mares, V. *Biol. Chem.*, 2004, 385(6), 557.
- [158] Stecca, B. A.; Nardo, B.; Chicco, P.; Mazziotti, A.; Bolondi, L.; Cavallari, A. *J. Hepatol.*, 1997, 27(2), 337.
- [159] Asada, Y.; Aratake, Y.; Kotani, T.; Marutsuka, K.; Araki, Y.; Ohtaki, S.; Sumiyoshi, A. *Histopathology*, 1993, 23(3), 265.
- [160] Wesley, U. V.; Tiwari, S.; Houghton, A. N. *Int. J. Cancer*, 2004, 109(6), 855.
- [161] Houghton, A. N.; Albino, A. P.; Cordon-Cardo, C.; Davis, L. J.; Eisinger, M. *J. Exp. Med.*, 1988, 167(1), 197.
- [162] Wesley, U. V.; Albino, A. P.; Tiwari, S.; Houghton, A. N. *J. Exp. Med.*, 1999, 190(3), 311.
- [163] Gonzalez-Gronow, M.; Grenett, H. E.; Gawdi, G.; Pizzo, S. V. *Exp. Cell Res.*, 2005, 303(1), 22.
- [164] Pethiyagoda, C. L.; Welch, D. R.; Fleming, T. P. *Clin. Exper. Metastasis*, 2001, 18(5), 391.
- [165] Frohman, L. A.; Downs, T. R.; Heimer, E. P.; Felix, A. M. *J. Clin. Invest.*, 1989, 83(5), 1533.
- [166] Van Gool, A. R.; Van Ojik, H. H.; Kruit, W. H.; Mulder, P. G.; Fekkes, D.; Bannink, M.; Scharpe, S.; Stoter, G.; Eggermont, A. M.; Maes, M.; Verkerk, R. *J. Interferon Cytokine Res.*, 2004, 24(7), 411.
- [167] Uematsu, T.; Urade, M.; Yamaoka, M.; Yoshioka, W. *J. Oral Pathol. Med.*, 1996, 25(9), 507.
- [168] Uematsu, T.; Urade, M.; Yamaoka, M. *J. Oral Pathol. Med.*, 1998, 27(3), 106.
- [169] Uematsu, T.; Tanaka, H.; Yamaoka, M.; Furusawa, K. *Anticancer Res.*, 2004, 24(2B), 619.
- [170] Kikkawa, F.; Kajiyama, H.; Shibata, K.; Ino, K.; Nomura, S.; Mizutani, S. *Biochim. Biophys. Acta*, 2005, 1751(1), 45.
- [171] Kajiyama, H.; Kikkawa, F.; Suzuki, T.; Shibata, K.; Ino, K.; Mizutani, S. *Cancer Res.*, 2002, 62(10), 2753.
- [172] Kajiyama, H.; Kikkawa, F.; Khin, E.; Shibata, K.; Ino, K.; Mizutani, S. *Cancer Res.*, 2003, 63(9), 2278.
- [173] Wilson, M. J.; Ruhland, A. R.; Quast, B. J.; Reddy, P. K.; Ewing, S. L.; Sinha, A. A. *J. Androl.*, 2000, 21(2), 220.
- [174] Johnson, R. C.; Zhu, D.; Augustin-Voss, H. G.; Pauli, B. U. *J. Cell Biol.*, 1993, 121(6), 1423.
- [175] Wesley, U. V.; McGroarty, M.; Homoyouni, A. *Cancer Res.*, 2005, 65(4), 1325.
- [176] Wilson, M. J.; Haller, R.; Li, S. Y.; Slaton, J. W.; Sinha, A. A.; Wasserman, N. F. *J. Urol.*, 2005, 174(3), 1124.
- [177] Droz, D.; Zachar, D.; Charbit, L.; Gogusev, J.; Chretein, Y.; Iris, L. *Am. J. Pathol.*, 1990, 137(4), 895.
- [178] Inamoto, T.; Yamochi, T.; Ohnuma, K.; Hosono, O.; Dang, N. H.; Morimoto, C. *Clin. Cancer Res.*, 2006, press.
- [179] Aratake, Y.; Kotani, T.; Tamura, K.; Araki, Y.; Kuribayashi, T.; Konoe, K.; Ohtaki, S. *Am. J. Clin. Pathol.*, 1991, 96(3), 306.
- [180] Kotani, T.; Aratake, Y.; Ogata, Y.; Umeki, K.; Araki, Y.; Hirai, K.; Kuma, K.; Ohtaki, S. *Cancer Lett.*, 1991, 57(3), 203.
- [181] Kotani, T.; Asada, Y.; Aratake, Y.; Umeki, K.; Yamamoto, I.; Tokudome, R.; Hirai, K.; Kuma, K.; Konoe, K.; Araki, Y.; et al. *J. Pathos.*, 1992, 168(1), 41.
- [182] Kotani, T.; Kawano, J.; Suganuma, T.; Hirai, K.; Umeki, K.; Aratake, Y.; Konoe, K.; Ohtaki, S. *Int. J. Exp. Pathos.*, 1992, 73(2), 215.
- [183] Tanaka, T.; Umeki, K.; Yamamoto, I.; Sakamoto, F.; Noguchi, S.; Ohtaki, S. *Int. J. Cancer*, 1995, 64(5), 326.
- [184] Kholova, I.; Ludvikova, M.; Ryska, A.; Hanzelkova, Z.; Cap, J.; Pecen, L.; Topolcan, O. *Neoplasma*, 2003, 50(3), 159.
- [185] Bauvois, B.; De Meester, I.; Dumont, J.; Rouillard, D.; Zhao, H. X.; Bosmans, E. *Br. J. Cancer*, 1999, 79(7-8), 1042.
- [186] Kondo, S.; Kotani, T.; Tamura, K.; Aratake, Y.; Uno, H.; Tsubouchi, H.; Inoue, S.; Niho, Y.; Ohtaki, S. *Leuk. Res.*, 1996, 20(4), 357.
- [187] Tsuji, T.; Sugahara, K.; Tsuruda, K.; Uemura, A.; Harasawa, H.; Hasegawa, H.; Hamaguchi, Y.; Tomonaga, M.; Yamada, Y.; Kamihira, S. *Int. J. Hematol.*, 2004, 80(3), 254.
- [188] Dang, N. H.; Aytac, U.; Sato, K.; O'Brien, S.; Melenhorst, J.; Morimoto, C.; Barrett, A. J.; Mouldrem, J. J. *Br. J. Haematol.*, 2003, 121(6), 857.
- [189] Jones, D.; Dang, N. H.; Duvic, M.; Washington, L. T.; Huh, Y. O. *Am. J. Clin. Pathol.*, 2001, 115(6), 885.
- [190] Aribi, A.; Keating, M.; O'Brien, S.; Ferrajoli, A.; Faderl, S.; Lerner, S.; Koller, C.; Wierda, W.; Cortes, J.; Kantarjian, H.; Ravandi-Kashani, F. *Blood*, 2005, 106(11), Abstract #5026.
- [191] Klobusicka, M.; Babusikova, O. *Neoplasma*, 1999, 46(5), 299.
- [192] Klobusicka, M.; Babusikova, O. *Gen. Physiol. Biophys.*, 1999, 18(Suppl. 1), 34.
- [193] Aldinucci, D.; Poletto, D.; Lorenzon, D.; Nanni, P.; Degan, M.; Olivo, K.; Rapana, B.; Pinto, A.; Gattei, V. *Clin. Cancer Res.*, 2004, 10(2), 508.
- [194] Ruiz, P.; Mailhot, S.; Delgado, P.; Amador, A.; Viciano, A. L.; Ferrer, L.; Zacharievich, N. *Cytometry*, 1998, 34(1), 30.
- [195] Bernengo, M. G.; Novelli, M.; Quaglino, P.; Lisa, F.; De Matteis, A.; Savoia, P.; Cappello, N.; Fierro, M. T. *Br. J. Dermatol.*, 2001, 144(1), 125.
- [196] Sokolowska-Wojdylo, M.; Wenzel, J.; Gaffal, E.; Steitz, J.; Roszkiewicz, J.; Bieber, T.; Tuting, T. *Clin. Exp. Dermatol.*, 2005, 30(6), 702.
- [197] Peranteau, W. H.; Endo, M.; Adibe, O. O.; Merchant, A.; Zoltick, P.; Flake, A. W. *Blood*, 2005, 106(11), Abstract #1275.
- [198] Korom, S.; De Meester, I.; Stadlbauer, T. H.; Chandraker, A.; Schaub, M.; Sayegh, M. H.; Belyaev, A.; Haemers, A.; Scharpe, S.; Kupiec-Weglinski, J. W. *Transplantation*, 1997, 63(10), 1495.
- [199] Christopherson, K. W., 2nd; Hangoc, G.; Mantel, C. R.; Broxmeyer, H. E. *Science*, 2004, 305(5686), 1000.
- [200] Christopherson, K. W.; Cooper, S.; Hangoc, G.; Broxmeyer, H. E. *Exp. Hematol.*, 2003, 31(11), 1126.
- [201] Christopherson, K. W., 2nd; Cooper, S.; Broxmeyer, H. E. *Blood*, 2003, 101(12), 4680.
- [202] Campbell, T. B.; Hangoc, G.; Broxmeyer, H. E. *Blood*, 2005, 106(11), Abstract #1708.

- [203] Christopherson, K. W.; Uralil, S. E.; Kidd, S. M.; Porecha, N. K.; Zabriskie, R. C. *Blood*, **2005**, *106*(11), Abstract #5220.
- [204] Boenig, H.; Priestley, G. V.; Papayannopoulou, T. *Blood*, **2005**, *106*(11), Abstract #471.
- [205] Kobayashi, S.; Ohnuma, K.; Uchiyama, M.; Iino, K.; Iwata, S.; Dang, N. H.; Morimoto, C. *Blood*, **2004**, *103*(3), 1002.
- [206] Kahne, T.; Neubert, K.; Faust, J.; Ansoerge, S. *Cell Immunol.*, **1998**, *189*(1), 60.
- [207] Steinbrecher, A.; Reinhold, D.; Quigley, L.; Gado, A.; Tresser, N.; Izikson, L.; Born, I.; Faust, J.; Neubert, K.; Martin, R.; Ansoerge, S.; Brocke, S. *J. Immunol.*, **2001**, *166*(3), 2041.
- [208] Wolf, G. B.; Scherberich, J. E.; Fischer, P.; Schoeppe, W. *Clinica Chimica Acta*, **1989**, *179*(1), 61.
- [209] Ho, L.; Aytac, U.; Stephens, L. C.; Ohnuma, K.; Mills, G. B.; McKee, K. S.; Neumann, C.; LaPushin, R.; Cabanillas, F.; Abbruzzese, J. L.; Morimoto, C.; Dang, N. H. *Clin. Cancer Res.*, **2001**, *7*(7), 2031.
- [210] Dong, R. P.; Tachibana, K.; Hegen, M.; Scharpe, S.; Cho, D.; Schlossman, S. F.; Morimoto, C. *Mol. Immunol.*, **1998**, *35*(1), 13.
- [211] Aytac, U.; Claret, F. X.; Ho, L.; Sato, K.; Ohnuma, K.; Mills, G. B.; Cabanillas, F.; Morimoto, C.; Dang, N. H. *Cancer Res.*, **2001**, *61*(19), 7204.
- [212] Aytac, U.; Sato, K.; Yamochi, T.; Ohnuma, K.; Mills, G. B.; Morimoto, C.; Dang, N. H. *Br. J. Cancer*, **2003**, *88*(3), 455.
- [213] Sato, K.; Aytac, U.; Yamochi, T.; Ohnuma, K.; McKee, K. S.; Morimoto, C.; Dang, N. H. *Br. J. Cancer*, **2003**, *89*(7), 1366.
- [214] Proost, P.; De Meester, I.; Schols, D.; Struyf, S.; Lambeir, A. M.; Wuyts, A.; Opdenakker, G.; De Clercq, E.; Scharpe, S.; Van Damme, J. *J. Biol. Chem.*, **1998**, *273*(13), 7222.
- [215] Nicholls, A.; Sharp, K. A.; Honig, B. *Proteins*, **1991**, *11*(4), 281.
- [216] Gorrell, M. D. *Clin. Sci. (Lond)*, **2005**, *108*(4), 277.
- [217] Chien, C. H.; Huang, L. H.; Chou, C. Y.; Chen, Y. S.; Han, Y. S.; Chang, G. G.; Liang, P. H.; Chen, X. *J. Biol. Chem.*, **2004**, *279*(50), 52338.
- [218] Bongers, J.; Lambros, T.; Ahmad, M.; Heimer, E. P. *Biochim. Biophys. Acta*, **1992**, *1122*(2), 147.
- [219] Hinke, S. A.; Pospisilik, J. A.; Demuth, H. U.; Mannhart, S.; Kuhn-Wache, K.; Hoffmann, T.; Nishimura, E.; Pederson, R. A.; McIntosh, C. H. *J. Biol. Chem.*, **2000**, *275*(6), 3827.
- [220] Proost, P.; Struyf, S.; Schols, D.; Opdenakker, G.; Sozzani, S.; Allavena, P.; Mantovani, A.; Augustyns, K.; Bal, G.; Haemers, A.; Lambeir, A. M.; Scharpe, S.; Van Damme, J.; De Meester, I. *J. Biol. Chem.*, **1999**, *274*(7), 3988.
- [221] Qiao, L.; Baumann, C. A.; Crysler, C. S.; Ninan, N. S.; Abad, M. C.; Spurlino, J. C.; Desjarlais, R. L.; Kervinen, J.; Neeper, M. P.; Bayoumy, S. S.; Williams, R.; Deckman, I. C.; Dasgupta, M.; Reed, R. L.; Huebert, N. D.; Tomczuk, B. E.; Moriarty, K. J. *Bioorg. Med. Chem. Lett.*, **2006**, *16*(1), 123.
- [222] Ahren, B.; Simonsson, E.; Larsson, H.; Landin-Olsson, M.; Torgeirsson, H.; Jansson, P.-A.; Sandqvist, M.; Bavenholm, P.; Efendic, S.; Eriksson, J. W.; Dickinson, S.; Holmes, D. *Diabetes Care*, **2002**, *25*(5), 869.
- [223] Sato, T.; Yamochi, T.; Aytac, U.; Ohnuma, K.; McKee, K. S.; Morimoto, C.; Dang, N. H. *Cancer Res.*, **2005**, *65*(15), 6950.

Knockdown of XAB2 Enhances All-*Trans* Retinoic Acid-Induced Cellular Differentiation in All-*Trans* Retinoic Acid-Sensitive and -Resistant Cancer Cells

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Abstract

Xeroderma pigmentosum group A (XPA)-binding protein 2 (XAB2) is composed of 855 amino acids, contains 15 tetratricopeptide repeat motifs, and associates with Cockayne syndrome group A and B proteins and RNA polymerase II, as well as XPA. *In vitro* and *in vivo* studies showed that XAB2 is involved in pre-mRNA splicing, transcription, and transcription-coupled DNA repair, leading to preimplantation lethality, and is essential for mouse embryogenesis. Retinoids are effective for the treatment of preneoplastic diseases including xeroderma pigmentosum and other dermatologic diseases such as photoaging. We therefore focused on defining the effect of XAB2 on cellular differentiation in the presence of ATRA treatment. In the present study, we showed that overexpression of XAB2 inhibited ATRA-induced cellular differentiation in human rhabdomyosarcoma cell line, and that knockdown of XAB2 by small interfering RNA (siRNA) increased ATRA-sensitive cellular differentiation in the human promyelocytic leukemia cell line HL60 at both physiologic (10^{-9} – 10^{-8} mol/L) and therapeutic (10^{-7} mol/L) concentrations of ATRA. Moreover, we found that XAB2 was associated with retinoic acid receptor α (RAR α) and histone deacetylase 3 in the nuclei. Finally, using siRNA against XAB2, we showed that the ATRA-resistant neuroblastoma cell line IMR-32 underwent cellular differentiation induced by ATRA at a therapeutic concentration (10^{-6} mol/L). These results strongly suggest that XAB2 is a component of the RAR corepressor complex with an inhibitory effect on ATRA-induced cellular differentiation and that XAB2 plays a role in ATRA-mediated cellular differentiation as an important aspect of cancer therapy. [Cancer Res 2007;67(3):1019–29]

Introduction

The retinoids are natural and synthetic derivatives of vitamin A that regulate a variety of important cellular functions such as differentiation and growth-suppressive effects in normal, prema-

lignant, and malignant cells (1, 2). These findings provided a basis for the use of retinoic acid in clinical cancer therapy and chemoprevention as differentiation therapy (3–6). For instance, all-*trans* retinoic acid (ATRA) has dramatically changed the clinical outcome of patients with acute promyelocytic leukemia from one that was highly lethal to one that now seems to be highly curable (7, 8). Acute promyelocytic leukemia is characterized by a chromosomal translocation, t(15;17), resulting in the fusion product PML-RAR α (*promyelocytic leukemia gene* on chromosome 15 and *retinoic acid receptor α gene* on chromosome 17; refs. 9–11). The PML-RAR α protein is stably associated with histone deacetylase (HDAC)-containing corepressor complexes, which leads to transcriptional repression of the program required for granulocytic differentiation of acute promyelocytic leukemia blasts at physiologic levels of retinoic acid (10^{-8} mol/L; refs. 12–14). At therapeutic concentrations (10^{-6} mol/L), retinoic acid dissociates the corepressor complex and recruits coactivator complexes, restoring the regulation of target genes (8, 11, 15).

Through metabolites such as ATRA, retinoids exert their effect on gene transcription mediated by the retinoic acid receptor (RAR) or retinoid X receptor (RXR) via the binding of RAR-RXR heterodimers or RXR-RXR homodimers to retinoic acid response elements (RARE; refs. 16–18). The ligand-dependent activation of genes via RAR or RXR depends on cofactor complexes interacting with RAR or RXR. In the absence of ATRA, RAR-RXR heterodimers interact with nuclear corepressors, such as silencing mediator of retinoid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (N-CoR), which recruit HDACs to induce chromatin modifications and transcriptional repression (18–20). Binding of retinoic acid permits dissociation of the corepressor complex and recruitment of coactivator complex, which in turn recruits histone acetyltransferase, thus leading to a permissive chromatin modification for opening promoter accessibility and finally to transcriptional activity (21). N-CoR/SMRT recruits HDAC3 to serve as effective corepressors of DNA-bound unliganded RAR-RXR (22, 23). Moreover, N-CoR and SMRT function not merely as platforms for recruitment of HDAC3 but also directly stimulate HDAC3 enzymatic activity (22, 24, 25). In acute promyelocytic leukemia, it has been reported that the PML-derived oligomerization domain of PML-RAR α is essential for such biological properties as enhanced binding of the corepressor N-CoR/SMRT with HDAC and blocking myeloid cell differentiation (26, 27). These molecular findings suggest that abnormal recruitment of HDACs by RAR complexes is a key pathogenic mechanism

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in acute promyelocytic leukemia, and that the repressor complexes, including HDACs itself, as well as RAR, are the molecular target of retinoic acid in differentiation therapy (28–32).

Xeroderma pigmentosum group A (XPA)-binding protein 2 (XAB2), which we independently isolated under the name of HDART⁷ by virtue of its sequence similarity to *Drosophila* CRN, is composed of 855 amino acids. XAB2 contains 15 tetratricopeptide repeat motifs and functions in a nucleotide excision repair complex (33). Cloned by virtue of its ability to interact with XPA protein in the yeast two-hybrid system, XAB2 thus derives its name as XPA-binding protein 2, and is found to form a complex with the nucleotide excision repair machinery (33). XAB2 knockout mice have been reported to show preimplantation lethality (34, 35). Xeroderma pigmentosum is a rare human hereditary disease characterized by hypersensitivity to sunlight, a high incidence of skin cancer in sun-exposed areas, and accelerated neurodegeneration (36, 37). Cells from xeroderma pigmentosum patients are hypersensitive to killing by UV irradiation, with early development of skin cancer being caused by defective DNA repair (38–41). Preneoplastic diseases including xeroderma pigmentosum were previously shown to be successfully treated with retinoids (1, 4). Expanding on these observations, we focus in this article on the role of XAB2 as a novel target for retinoic acid-based differentiation therapy for cancers, defining in particular the biological and physiologic function of XAB2 in the RAR/HDAC3 complex. We now show that XAB2 functions as a corepressor of the RAR/HDAC3 complex, and that knockdown of XAB2 by small interfering RNA (siRNA) enhances sensitivity to ATRA in the acute promyelocytic leukemia cell line HL60 and the rhabdomyosarcoma cell line MM-1-19P, as well as the ATRA-resistant neuroblastoma cell line IMR-32.

Materials and Methods

Cells, antibodies, and reagents. HEK293T, HepG2, HL60, and MM-1-19-P were grown in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 100 units/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). MM-1-19-P human rhabdomyosarcoma cell line, HL60 human promyelocytic leukemia cell line, and retinoic acid-resistant human neuroblastoma cell line IMR-32 were grown in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin, using Laminin six-well multiwell plates (BD Biosciences, San Jose, CA).

The following antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-Flag M2 monoclonal antibody (mAb), anti-green fluorescent protein (GFP) polyclonal antibody (pAb), antitubulin mAb, anti-HDAC1 pAb, anti-HDAC3 pAb, anti-HDAC4 pAb, anti-HDAC6 pAb, anti-RAR α pAb, Texas red-conjugated antigoat immunoglobulin secondary antibody, Texas red-conjugated antimouse immunoglobulin secondary antibody, and FITC-conjugated antimouse immunoglobulin secondary antibody. Anti-XAB2 mAb was purchased from Abnova (Taipei, Taiwan); anti-XAB2 polyclonal antibody was obtained from Mo Bi Tec (Goettingen, Germany); phycoerythrin-conjugated CD11b and 7-amino-actinomycin D were obtained from BD Pharmingen (San Jose, CA); and anti-acetyl-histone H4 polyclonal antibody was obtained from Upstate (Chicago, IL).

siRNA against human XAB2. We selected the target sequences from +90 to +108 and +1,673 to +1,691 downstream of the start codon of human XAB2 mRNA (National Center for Biotechnology Information accession no. NM_020196; sense siRNA ss1, 5'-GAACCAUUCUCUGUCAAAdTdT-3'; sense

siRNA ss2, 5'-GUAUUTAUTGAUAAAATdTdT-3'). Moreover, control siRNA was prepared to examine nonspecific effects of siRNA duplexes (5'-GAACACUACUUCGUCAAAdTdT-3'). These selected sequences also were submitted to a BLAST search against the human genome sequence to ensure that only one gene of the human genome was targeted. siRNAs were purchased from Japan Bioservice (Saitama, Japan). siRNA duplex formation (annealing) was done as previously described (42). A total of 60 pmol of siRNA duplexes were transfected into 0.5×10^6 cells using Nucleofector (Amaxa, Inc., Gaithersburg, MD). After 48 h of transfection, cells were prepared for examination.

Cellular differentiation. MM-1-19-P cells (1×10^6 /mL) were transfected with 2 µg of pcDNA3-XAB2 or pcDNA3 plasmids as mock control, and pEGFP-C1 plasmid (0.4 µg) was cotransfected for the identification of transfected cells. After 24 h of transfection, cells were treated with 2 µmol/L ATRA for an additional 24 h and were submitted to fluorescence microscopy analysis. The effect of exogenous XAB2 on cells was observed by morphologic analysis of cells through the use of phase-contrast microscopy merged with green fluorescence of GFP. Myocytic differentiation of rhabdomyosarcoma cells was observed with phase-contrast microscopy, with two pathologists identifying the morphologic changes to myocytic differentiation. For quantification, the percentage of GFP-positive, myocyte-differentiated cells among a total of 1,000 GFP-positive cells was shown in the graph. All experiments were done in triplicate and the results presented as means with SE.

After transfection of siRNA, IMR-32 cells (1×10^6 /mL) were incubated in the presence of 2 µmol/L ATRA for an additional 48 h. Neuroganglionic differentiation of neuroblastoma cells was observed as previously described (43); cells with extending processes longer than twice the diameter of the cell body were counted as being differentiated, with two pathologists identifying the morphologic changes to formation of neurite-like outgrowth. For quantification, the percentage of differentiated cells among 1,000 cells was shown in the graph. All experiments were done in triplicate and the results presented as means with SE.

Relative quantitative reverse transcription-PCR assay. In experiments assessing expression of RAR β and CYP26 mRNA, after HEK293T or HL60 cells were treated as described above, total RNA was then extracted and cDNA was produced as previously described (44). PCR primers for the coding sequences of RAR β and CYP26 were as follows: RAR β , forward 5'-ATGTTTGACTGTATGGATGTT-3', reverse 5'-CCCACTTCAAAGCACTTCTG-3'; CYP26, forward 5'-GATGAAGCGCAGGAAATAC-3', reverse 5'-ATGGC-GATTCCGGAACATGGAG-3'. These primers were designed for the coding sequences that are located on different exons to eliminate amplifying genomic DNA contamination in the PCRs. The quantities of mRNA were adjusted equally by using PCR of β -actin (forward primer 5'-CAAGA-GATGGCCACGGCTGCT-3' and reverse primer 5'-TCCTTCTGCATCC-TGTCGGCA-3' as the internal control).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation assay was done using 1×10^6 of HL60 cells in the presence or absence of 1 µmol/L ATRA for 72 h. Protein-DNA complexes were prepared using EZ ChIP Chromatin Immunoprecipitation Kit (Upstate) according to the manufacturer's instruction. After washing and reverse cross-linking, immunoprecipitated DNA was purified and PCR was done. The following PCR primers were used to detect the immunoprecipitated promoter DNA: RAR β promoter, forward 5'-CTCTGGCTGTCTGCTTTTGC-3', reverse 3'-CAAAAAGCCTTCCGAATGC-3'; CYP26 promoter, forward 5'-TAAAGATTTTGGGCAGCG-3', reverse 5'-CATCTGCAAAGGTTTCCCCAA-3'. The PCR reactions were done as described above.

Coimmunoprecipitation assay. For coimmunoprecipitation assays involving endogenous XAB2 and HDACs, nuclear extracts were prepared from 1×10^7 HL60 cells as previously described (45). Nuclear extracts were then isolated by centrifugation at $15,000 \times g$ for 30 min. Immunoprecipitation assays were done subsequently by incubating nuclear extracts with 2 µg of control immunoglobulin and protein G-Sepharose beads (Amersham Pharmacia, Piscataway, NJ) at 4°C for 1 h. After centrifugation, supernatants were incubated with 2 µg of anti-XAB2 mAb at 4°C for 1 h, followed by the addition of protein G-Sepharose beads for 1 h (45). Cell lysates and nuclear extracts were prepared and coimmunoprecipitation assay, SDS-PAGE, and

⁷ Unpublished observation.

Western blot analysis were conducted according to the methods described elsewhere (44, 45).

Confocal laser microscopy. For fluorescence microscopy experiments, HL60 cells were incubated for 24 h in the presence or absence of ATRA and then attached to microslide glass (Matsunami Glass, Inc., Tokyo, Japan) coated with poly-L-lysine and fixed with ice-cold acetone/methanol (1:1). After washing with ice-cold PBS, cells were blocked with mouse and goat immunoglobulin isotypes, followed by incubation with anti-XAB2 mAb, RAR α pAb, or anti-HDAC3 pAb (each at a concentration of 10 μ g/mL), then washed and incubated with FITC-conjugated antimouse immunoglobulin and/or Texas red-conjugated antigoat immunoglobulin antibodies (each at a concentration of 5 μ g/mL). Confocal microscopy was done as described elsewhere (44, 45).

Chloramphenicol acetyltransferase assay. To examine the potential effect of XAB2 on ATRA-mediated transcriptional activity, HepG2 cells were transiently transfected with pRARE-chloramphenicol acetyltransferase (CAT), which is a RARE-containing RAR reporter plasmid, cloned in frame with CAT under the control of a thymidine kinase minimal promoter. Cells were seeded onto 35-mm-diameter dishes at a density of 1.75×10^5 per well and incubated overnight in FCS-supplemented medium. The following day, cells were transfected with pRARE-CAT (1 μ g), pSV- β -gal (0.5 μ g), and pcDNA3-XAB2 (0.5 or 1.0 μ g), or pcDNA3 as mock control. The amount of expression vector was adjusted to 1 μ g with pcDNA3 empty vector in each transfection. Twenty-four hours later, culture medium was removed and cells were washed thrice with serum-free MEM (Life Technologies), and then treated with fresh medium containing 100 nmol/L ATRA. Cells were harvested after 24 h and assayed for β -galactosidase and CAT activity with the use of the β -Galactosidase Enzyme Assay System (Promega, Madison, WI) and CAT Enzyme Assay System (Promega) according to the manufacturer's instruction. For transfection efficiency, CAT activity was normalized to β -galactosidase activity. The CAT activity in control cells treated with DMSO vehicle and a mock vector was set as equal to one and all other values are expressed as fold induction. Data represent mean from triplicate experiments \pm SE.

Statistics. Student's *t* test was used to determine whether the difference between control and experimental samples was significant ($P < 0.05$).

Results

Overexpression of XAB2 inhibits cellular differentiation and transcriptional activation induced by ATRA. To determine an effect of XAB2 on cellular differentiation, we first examined whether cellular differentiation is induced by overexpression of exogenous XAB2. For this purpose, we used the human rhabdomyosarcoma cell line MM-1-19-P, which is mainly composed of small polygonal cells and terminally differentiates to myotube-like giant cells following treatment with ATRA (46). As shown in Fig. 1A, MM-1-19-P cells transfected with mock vector differentiated to myotube-like giant cells following treatment with ATRA (arrowheads in *a*). On the other hand, ATRA did not induce any morphologic changes in MM-1-19-P cells transfected with XAB2 expression plasmid (Fig. 1A, *b*). For further confirmation and quantification of ATRA-induced cellular differentiation of MM-1-19-P cells, we next conducted cotransfection experiments with XAB2-expressing plasmids (pcDNA3-XAB2) or mock plasmid (pcDNA3) and GFP-expressing plasmids (pEGFP-C1) for visualization. As shown in Fig. 1B, no change was observed both in MM-1-19-P cells transfected with mock and GFP-expressing plasmids and in MM-1-19-P cells transfected with XAB2- and GFP-expressing plasmids (*a* and *c*). On the other hand, myocytic cellular differentiation following treatment with ATRA was significantly observed in mock-transfected MM-1-19-P cells, which were cotransfected with GFP-expressing plasmid (Fig. 1B, *b*). However, cellular differentiation to myotube-like giant cell induced by ATRA was not detected in MM-1-19-P cells transfected with XAB2

expression plasmid (Fig. 1B, *d*). To quantify the number of ATRA-induced differentiated cells in the mock- and XAB2-transfected MM-1-19-P cell population, we counted the myotube-like giant cells expressing GFP under examination by fluorescence and bright-field microscopy. As shown in Fig. 1C, MM-1-19-P cells transfected with mock and GFP vectors exhibited an increase in the number of differentiated cells (Fig. 1C, #). However, ATRA-induced differentiation of XAB2-transfected MM-1-19-P cells was significantly inhibited (Fig. 1C, ##). These results suggest that XAB2 has an inhibitory effect on ATRA-mediated cellular differentiation.

To confirm the inhibitory effect of XAB2 on ATRA-induced cell differentiation, CAT assays were done with the RAR reporter plasmid containing a RARE under the control of a thymidine kinase minimal promoter (pRARE-CAT). HepG2 cells were transiently transfected with pcDNA3-XAB2, which allows for the constitutive expression of XAB2, and were grown in the presence or absence of ATRA. In mock (pcDNA3)-transfected cells, CAT activity was induced 5-fold by ATRA. However, the ATRA-induced CAT activation was suppressed in a dose-dependent manner by XAB2 (Fig. 1D). These results suggest that XAB2 suppresses ATRA-activated transcription.

To further determine whether the overexpressed XAB2 protein plays an inhibitory role in ATRA-induced activation of transcription, we analyzed ATRA-targeted gene expression pattern in HEK293T cells by reverse transcription-PCR (RT-PCR). For this purpose, two RAR α target genes, RAR β and CYP26, were selected (47). HEK293T cells were transiently transfected with pcDNA3-XAB2 to allow for the constitutive expression of XAB2 and were grown in the presence or absence of ATRA. Total RNA was purified and RT-PCR using RAR β -, CYP26-, and β -actin-specific primers was done. As shown in Fig. 1E, in mock (pcDNA3)-transfected cells, endogenous RAR β and CYP26 mRNA expression was induced by ATRA (lanes 1 and 2). However, the ATRA-induced RAR β and CYP26 expression was suppressed in a dose-dependent manner by XAB2 (Fig. 1E, lanes 3–6). Exogenous XAB2 transfection did not affect the expression level of endogenous RAR β and CYP26 mRNA (Fig. 1E, lanes 1, 3, and 5). These results confirm our earlier data indicating that XAB2 suppresses ATRA-activated gene transcription.

Knockdown of XAB2 by siRNA enhances cellular differentiation of HL60 cells induced by ATRA. To further define an inhibitory effect of XAB2 on ATRA-induced cellular differentiation, we next used the human promyelocytic leukemia cell line HL60, which is induced to differentiate to neutrophilic cells by various drugs including ATRA (48). For this purpose, we prepared two separate siRNA against XAB2, as described in Materials and Methods, to ensure the specificity of the knockdown effect. As shown in Fig. 2A, these siRNAs against XAB2 (ss1 and ss2) effectively knocked down XAB2 expression in HL60 cells (*top*, lanes 2 and 4). Because XAB2 in HL60 cells was not significantly knocked down by control siRNA (Fig. 2A, *top*, lanes 1 and 3) and expression level of tubulin as a housekeeping protein was not affected by both of siRNAs (Fig. 2A, *bottom*), this inhibitory effect of XAB2 by siRNAs in HL60 cells was specific. In addition, the expression levels of HDAC3 and RAR α were not changed in the presence or absence of siRNA treatment (data not shown). We next examined ATRA-induced cellular differentiation of HL60 cells in the presence or absence of siRNAs against XAB2 through flow cytometric analysis of cell-surface expression level of CD11b (49). As shown in Fig. 2B, ATRA-induced HL60 cell differentiation was enhanced in a dose-dependent manner with treatment with siRNAs against XAB2 as compared with the level of differentiation

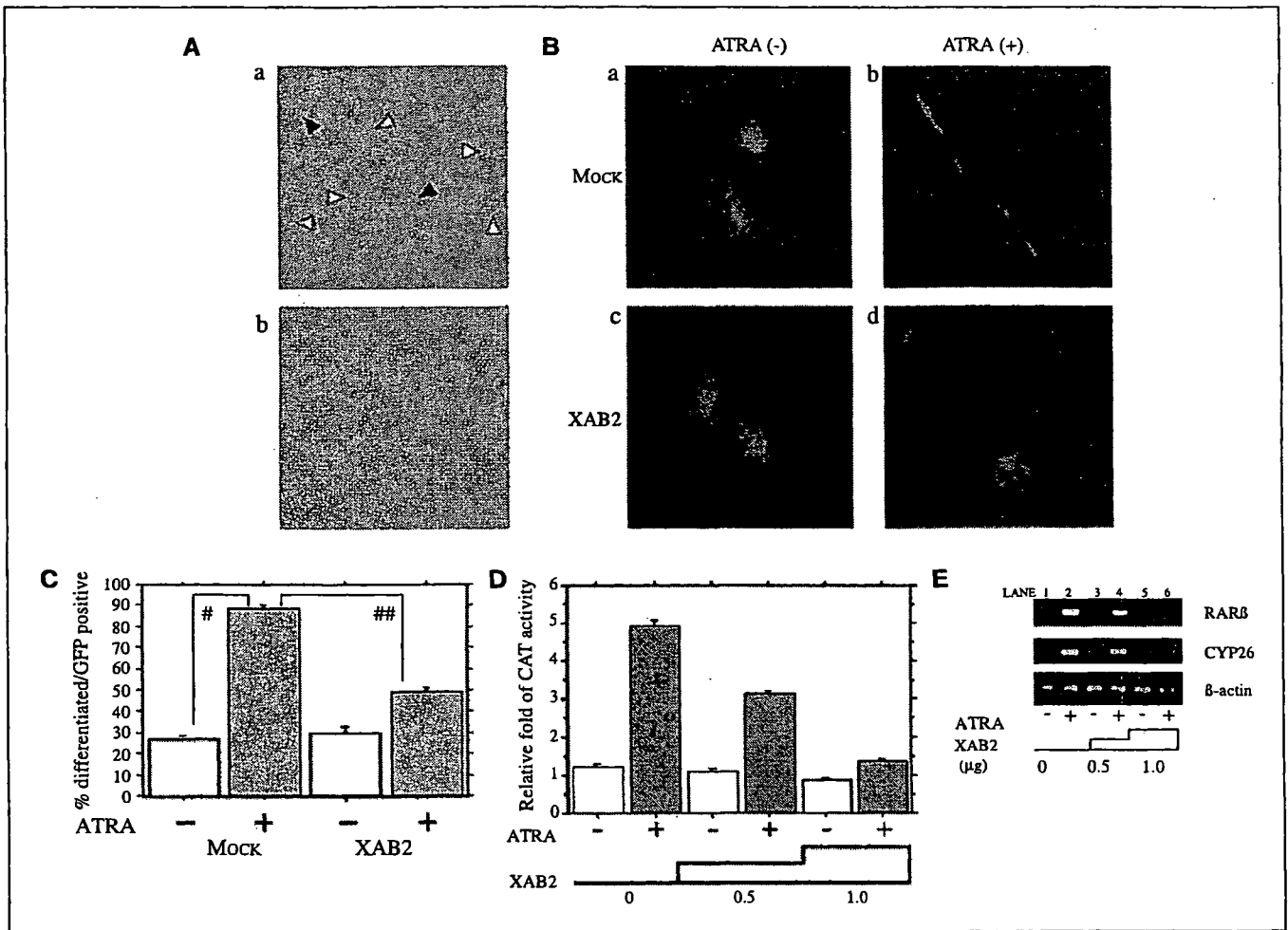


Figure 1. XAB2 inhibits ATRA-induced cellular differentiation and transcriptional activation. **A**, ATRA-sensitive human rhabdomyosarcoma MM-1-19-P cells were transiently transfected with pcDNA3 as a vector control (*a*) or pcDNA-XAB2 (*b*). Twelve hours later, the transfection medium was replaced with fresh media containing 2 μmol/L ATRA. After 24-h incubation, cells were visualized with phase-contrast microscopy. *Black* and *white arrowheads*, differentiated cells that form myotube-like processes. **B**, MM-1-19-P cells were transfected with 2 μg of pcDNA3 (*a* and *b*) or pcDNA3-XAB2 (*c* and *d*) and cotransfected with 0.4 μg of pEGFP-C1 to identify transfected cells (*a-d*). Cells were treated with DMSO vehicle as a control (*a* and *c*) or with ATRA (*b* and *d*) by the same method as described in (*A*) and visualized by fluorescence microscopy. Because the amount of pcDNA3-XAB2 transfected is five times more than that of pEGFP-C1, GFP-positive cells definitely contains exogenous XAB2. **C**, percentage of morphologically differentiated GFP-positive cells among 1,000 GFP-positive cells as shown in (*A*) and (*B*). *Columns*, mean from three independent experiments; *bars*, SE. # and ##, significant changes compared with control ($P < 0.05$). **D**, HepG2 cells were transiently transfected with pRARE-CAT (2 μg), pSV-β-gal (0.4 μg), and pcDNA3-XAB2 (0, 0.5, or 1.0 μg). The amount of expression vector was adjusted to 1 μg of pcDNA3 empty vector in each transfection. Twenty-four hours after transfection, cells were treated with 100 nmol/L ATRA or DMSO vehicle as a control. Cells were then harvested after 24 h and assayed for β-galactosidase and CAT activity. For transfection efficiency, CAT activity was normalized to β-galactosidase activity. The CAT activity in control cells treated with DMSO vehicle and a mock vector was set as equal to one and all other values are expressed as fold induction. *Columns*, mean from triplicate experiments; *bars*, SE. **E**, HEK293T cells were transiently transfected with pcDNA3 empty or pcDNA3-XAB2 vector (0, 0.5, or 1.0 μg). The amount of expression vector was adjusted to 1 μg of pcDNA3 empty vector in each transfection. Twenty-four hours after transfection, cells were treated with 1 μmol/L ATRA or DMSO vehicle as a control. Cells were then harvested after 24 h and total RNA was extracted. RT-PCR assays using primers for RARβ, CYP26, and β-actin (as internal control) were then done. All experiments were done at least thrice with similar results.

observed in HL60 cells treated with control siRNA. These data suggest that specific knockdown of XAB2 enhances ATRA-induced cellular differentiation at physiologic levels (10^{-9} - 10^{-8} mol/L) as well as at therapeutic levels (10^{-7} mol/L).

To further confirm whether knockdown of XAB2 enhances ATRA-induced gene expression, we analyzed ATRA-targeted gene expression pattern in HL60 cells by RT-PCR, in the presence or absence of siRNA against XAB2. As shown in Fig. 2C, ATRA-induced mRNA expression of RARβ and CYP26 was increased in a dose-dependent manner (*lanes 1, 4, 7, and 10*). The increased expression of RARβ and CYP26 mRNA was further enhanced in HL60 cells treated with siRNA against XAB2 (Fig. 2C, *lane 4* versus *lanes 5* and *6*, *lane 7* versus *lanes 8* and *9*, and *lane 10* versus *lanes 11* and *12*). This enhanced expression of RARβ and CYP26

mRNA was again observed to be dependent on ATRA doses (Fig. 2C, *lanes 5* and *6*, *8* and *9*, and *11* and *12*). These data suggest that specific knockdown of XAB2 enhances not only cellular differentiation but also induction of ATRA-targeted genes at physiologic (10^{-9} - 10^{-8} mol/L) and therapeutic (10^{-7} mol/L) levels.

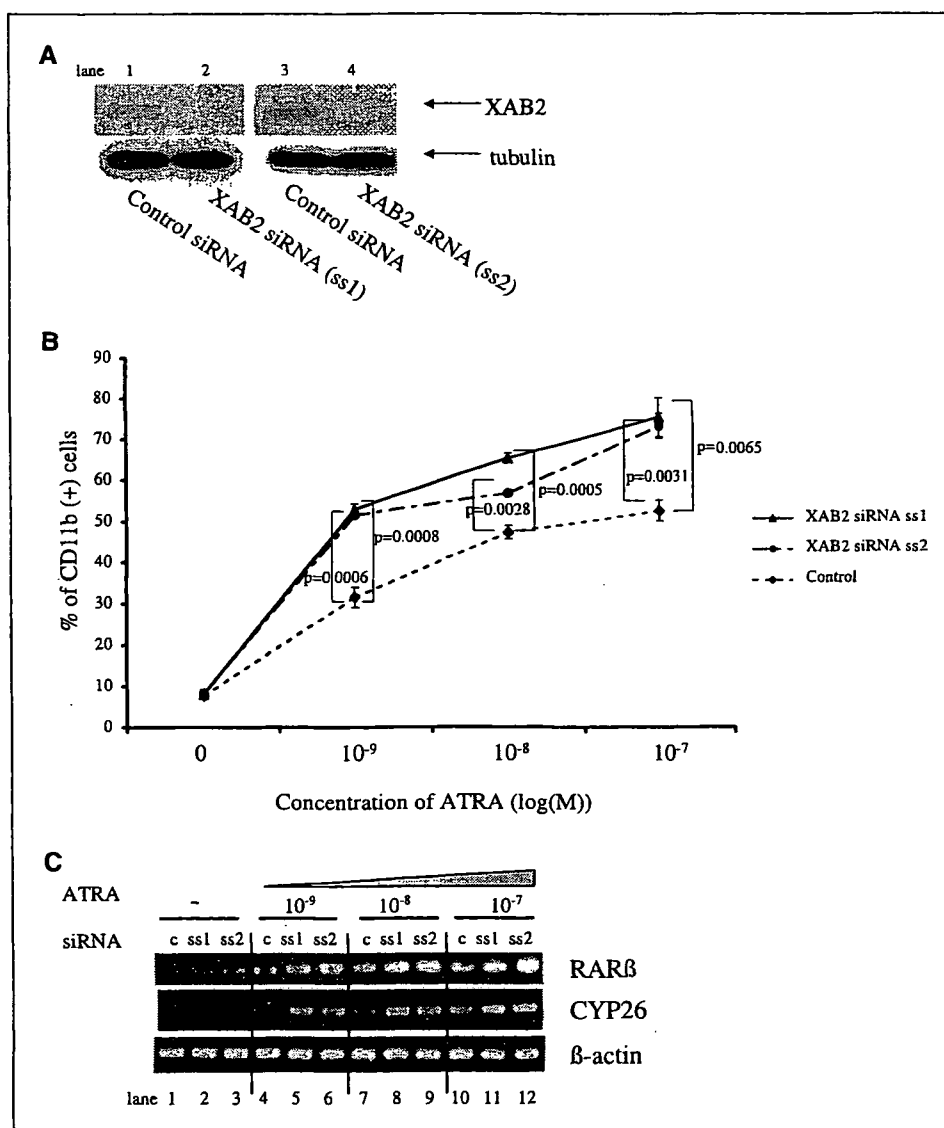
XAB2 is associated with HDAC3. Retinoid-dependent cellular differentiation of embryonal carcinoma cell lines was previously shown to be mediated by HDAC activity (12, 15, 50). We thus examined whether XAB2 involvement in retinoid mediated-transcriptional regulation is through HDAC activity. For this purpose, the potential association of XAB2 with HDAC activity on RAR-regulated transcription was examined via RARE-chloramphenicol acetyltransferase (CAT) assays, which were done on the RAR reporter plasmid containing a RARE under the control of

thymidine kinase minimal promoter (pRARE-CAT). HepG2 cells were transiently transfected with pcDNA3-XAB2 to allow for the constitutive expression of XAB2 and were then grown in the presence or absence of ATRA. As shown in Fig. 3A, transcriptional activity was significantly enhanced by ATRA addition (Fig. 3A, #), and this ATRA-induced transcriptional activity was decreased in the presence of overexpression of XAB2 (Fig. 3A, ##). Moreover, the addition of trichostatin A or sodium butyrate, specific inhibitors of HDAC, completely antagonized the repression by XAB2 of RAR reporter activity (Fig. 3A, ###). To further confirm the antagonistic effect of HDAC inhibitors on XAB2-mediated repression, we analyzed the expression level of endogenous retinoic acid-targeted genes *RARβ* and *CYP26*. As shown in Fig. 3B, the mRNA level of *RARβ* and *CYP26* was enhanced by ATRA addition (lane 2), and this ATRA-induced enhancement of *RARβ* and *CYP26* mRNA expression was decreased by XAB2 overexpression (lane 3). In contrast, the addition of trichostatin A or sodium butyrate completely antagonized the repression by XAB2 of ATRA-induced *RARβ* and *CYP26* mRNA expression (Fig. 3B, lanes 4 and 5).

These data suggest that the XAB2-mediated inhibitory effect of ATRA-induced transcriptional activity is associated with HDAC activity.

To further explore XAB2 association with HDACs, we next evaluated the potential interaction of XAB2 with HDACs in a transcriptional component through coimmunoprecipitation assays. HEK293T cells were transfected with an expression plasmid of GFP-XAB2 alone or together with expression plasmids of Flag-HDAC1, HDAC3, HDAC4, and HDAC6 (Fig. 3C, top). The Flag-HDACs were immunoprecipitated with antibody against the Flag tag, and coimmunoprecipitated GFP-XAB2 protein was detected by Western blotting with anti-GFP antibody. As shown in Fig. 3C, the GFP-XAB2 protein was coimmunoprecipitated with Flag-HDAC1, HDAC3, HDAC4, and HDAC6 from HEK293T cell lysates (middle, lanes 1 and 3-5). No cellular protein was detected by the anti-GFP antibody in immunoprecipitants of lysates of HEK293T cells transfected with Flag-vector control plasmid (middle, lane 2). The expression level of GFP-XAB2 was not affected by the presence or absence of Flag-HDACs (Fig. 3C, bottom).

Figure 2. Knockdown of XAB2 by siRNA enhances ATRA-mediated cellular differentiation of HL60 cells. **A**, HL60 cells were transfected with sense siRNAs of XAB2 gene (*XAB2 siRNA ss1* or *ss2*) or with control nonsense siRNA (*Control siRNA*) using Nucleofector. Cell lysates were resolved by 10% SDS-PAGE and immunoblotted with anti-XAB2 mAb, followed by stripping and reprobing with antitubulin mAb. All experiments were done at least thrice with similar results. **B**, effect of siRNAs against XAB2 on the induction of HL60 cell differentiation by ATRA. Following transfection with siRNA (*XAB2 siRNA ss1*, *ss2*, or control siRNA), HL60 cells were treated with ATRA at the indicated concentrations and incubated for 24 h. CD11b expression was analyzed by flow cytometry as described in Materials and Methods. Points, mean values calculated from three independent experiments; bars, SE. Percent values were percent of CD11b-positive cell number in 7-amino-actinomycin D-negative viable cells. **C**, following transfection with siRNA (*XAB2 siRNA ss1*, *ss2*, or control siRNA), HL60 cells were treated with ATRA at the indicated concentrations, or with DMSO vehicle as a control, and incubated for 24 h. Total RNA was extracted and RT-PCR assays using primers for *RARβ*, *CYP26*, and β -actin (as internal control) were then done. All experiments were done at least thrice with similar results.



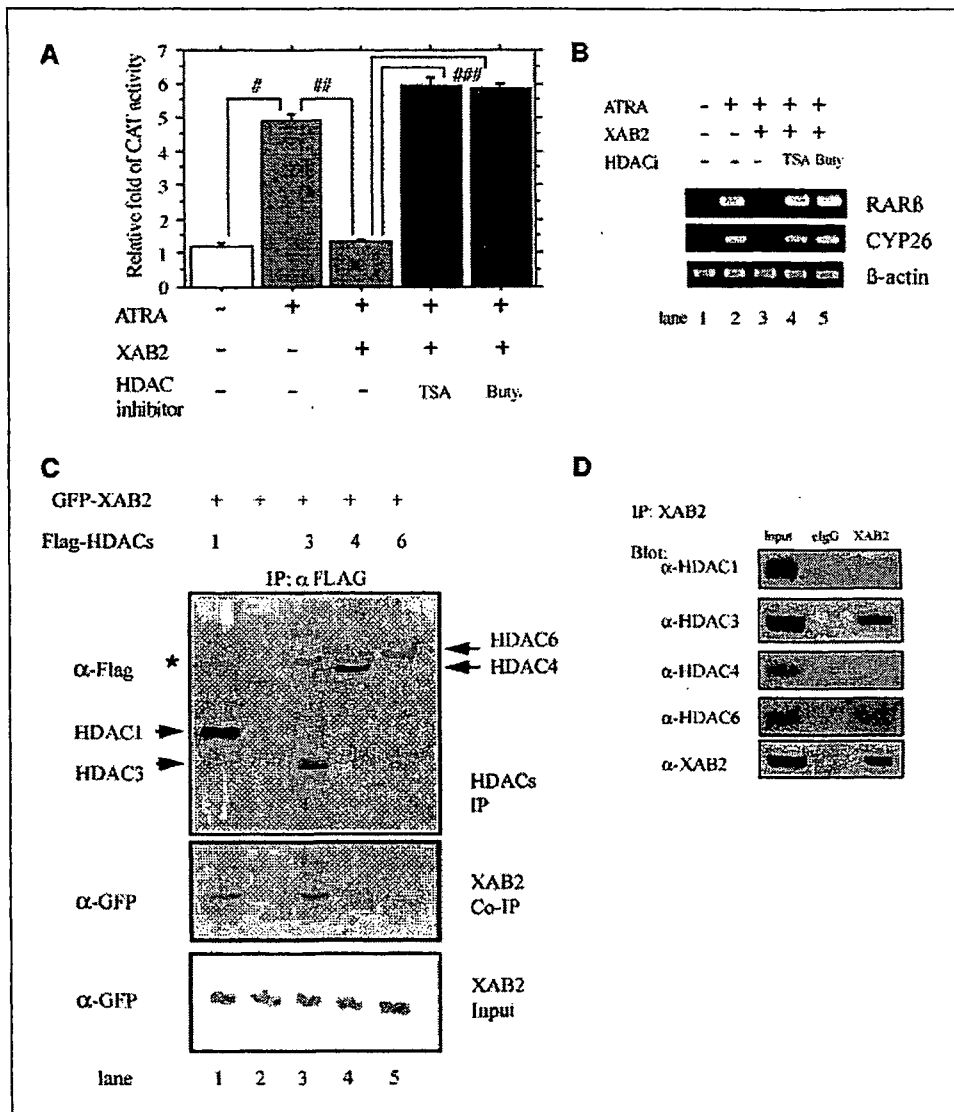


Figure 3. XAB2 is associated with HDAC3. **A**, HepG2 cells were transiently transfected with pRARE-CAT (2 μg), pSV-β-gal (0.4 μg), and pcDNA3-XAB2 (1.0 μg) or pcDNA3 empty vector (1.0 μg). Twenty-four hours after transfection, cells were treated with 100 nmol/L ATRA or DMSO vehicle as a control in the presence or absence of trichostatin A (TSA; 10 nmol/L) or sodium butyrate (Buty; 1 mmol/L). Cells were then harvested after 24 h and assayed for β-galactosidase and CAT activity. For transfection efficiency, CAT activity was normalized to β-galactosidase activity. CAT activity in control cells treated with DMSO vehicle and a mock vector was set as equal to one and all other values are expressed as fold induction. Columns, mean from triplicate experiments; bars, SE. #, ##, and ###, significant changes compared with control ($P < 0.05$). **B**, HEK293T cells were transiently transfected with pcDNA3 empty or pcDNA3-XAB2 vector (1.0 μg). Twenty-four hours after transfection, cells were treated with 1 μmol/L ATRA or DMSO vehicle as a control in the presence or absence of trichostatin A (10 nmol/L) or sodium butyrate (1 mmol/L). Cells were then harvested after 24 h and total RNA was extracted. RT-PCR assays using primers for RARβ, CYP26, and β-actin (as internal control) were then done. All experiments were done at least thrice with similar results. **C**, HEK293T cells were transfected with pEGFP-C1-XAB2 and pFLAG-CMV-HDACs (lane 1, HDAC1; lane 3, HDAC3; lane 4, HDAC4; lane 5, HDAC6). pFLAG-CMV empty vector was transfected along with pEGFP-C1-XAB2 (lane 2). After 24-h transfection, cells were harvested and nuclear extracts were immunoprecipitated with anti-Flag M2 mAb and then Western blotted with anti-Flag M2 mAb (top) or anti-GFP polyclonal antibody (middle). Ten micrograms of nuclear extracts were Western blotted with anti-GFP polyclonal antibody to show equal expression of transfected GFP-XAB2 protein (bottom). Asterisk, nonspecific bands. **D**, in the absence of ATRA, nuclear extracts were prepared from HL60 cells and immunoprecipitation (IP) assays were conducted with anti-XAB2 antibody or control immunoglobulin (clgG). Immunoprecipitation complexes, along with 10% input of nuclear extract, were then separated by 4% to 20% SDS-PAGE, followed by Western blotting with indicated antibodies. Representative results obtained from three independent experiments.

Various HDACs have previously been reported to be involved in RARα-mediated transcriptional regulation via interaction with N-CoR/SMRT (20, 51, 52). However, the identity of the precise HDACs involved remains controversial, partly because of the different cell types or assay systems being used. We therefore attempted to determine the specific HDACs among HDAC1, HDAC3, HDAC4, or HDAC6 that are associated with XAB2 in our system. For this purpose, immunoprecipitation assays detecting endogenous HDACs and XAB2 proteins were done on nuclear

extract of HL60 cells. As shown in Fig. 3D, endogenous HDAC1, HDAC3, HDAC4, HDAC6, and XAB2 were expressed in HL60 cells (lane 1), with each control immunoglobulin G (IgG) not able to precipitate endogenous HDAC1, HDAC3, HDAC4, HDAC6, and XAB2 (lane 2). In this experimental system, HDAC3 was clearly detected in the complex precipitated by XAB2 whereas very slight amount of HDAC1 was coprecipitated with XAB2. On the other hand, no portion of HDAC4 or HDAC6 was detected in the complex (lane 2). These data indicate that XAB2 is associated mainly with

HDAC3 in our system and that XAB2 associates with HDAC3-dependent transcriptional components that interact with RARE.

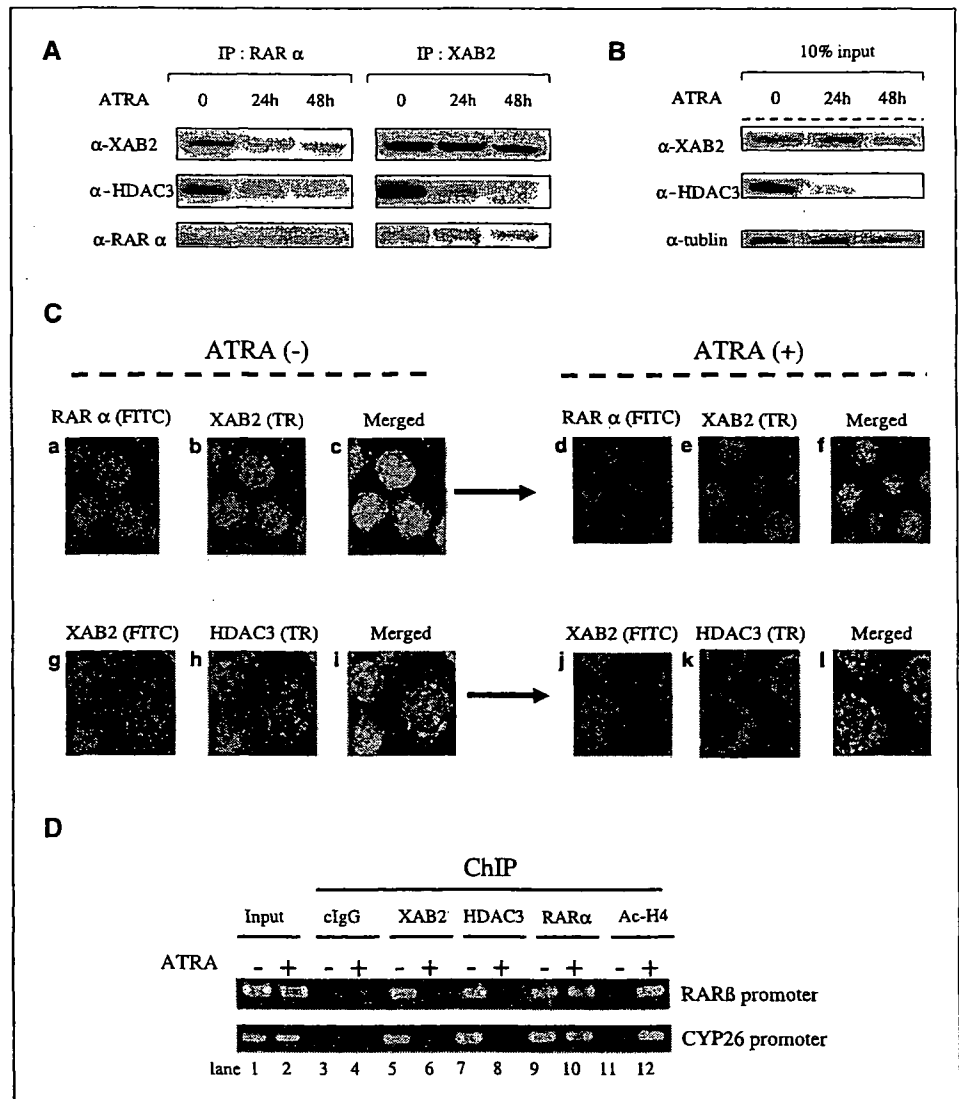
ATRA leads to dissociation of XAB2 from RAR α and HDAC3. As shown above, XAB2 is associated mostly with HDAC3 and is involved in the suppression of ATRA-mediated cellular differentiation. We next examined potential alterations in the complex containing endogenous XAB2, RAR α , and HDAC3 after ATRA treatment. For this purpose, we conducted immunoprecipitation experiments using nuclear extracts of HL60 cells incubated in the presence or absence of ATRA. As shown in Fig. 4A, XAB2 was found with RAR α and HDAC3 in immunoprecipitation complexes of nuclear extracts of untreated cells (Fig. 4A, lanes 1 and 4). Following treatment of HL60 cells with ATRA for 24 to 72 h, XAB2 and HDAC3 were dissociated from RAR α (Fig. 4A, lanes 2 and 3) and, similarly, RAR α and HDAC3 were dissociated from XAB2 (Fig. 4A, lanes 5 and 6). At these time points, Western blot analysis of nuclear extracts revealed that the expression level of HDAC3 was decreased by ATRA treatment (Fig. 4B, middle), whereas the expression levels of XAB2 and tubulin were not changed. These data indicate that XAB2 forms a complex with RAR α and HDAC3

and that, following ATRA treatment, XAB2 is disengaged from the complex without degradation while HDAC3 is degraded, as previously reported (22).

To further characterize the association among XAB2, HDAC3, and RAR α , we did fluorescence microscopic analysis to determine the subcellular localization of these structures. As shown in Fig. 4C, XAB2 clearly colocalized with RAR α , as well as HDAC3, in the nuclei of untreated HL60 cells (*a-c* and *g-i*). Following treatment of HL60 cells with ATRA, XAB2 was not found to colocalize with RAR α (*d-f*). Moreover, whereas XAB2 was localized in the nuclei after ATRA treatment (*e* and *j*), HDAC3 was not detected in the nuclei after ATRA treatment (*k*). Taken together, these data indicate that XAB2 associates with HDAC3 and RAR α in the nuclei of untreated HL60 cells, with ATRA treatment resulting in the subsequent disengagement of XAB2 and HDAC3 from the complex. Furthermore, ATRA treatment induces change of localization of XAB2 in the nuclei while HDAC3 is degraded as reported previously (22).

To further confirm that XAB2, RAR α , and HDAC3 bind to the same ATRA-targeted gene promoters and that XAB2 and HDAC3

Figure 4. XAB2 forms a complex with RAR α and HDAC3 and is dissociated from the complex following ATRA treatment. **A**, nuclear extracts of HL60 cells were immunoprecipitated with anti-RAR α polyclonal antibody or anti-XAB2 mAb in the presence or absence of ATRA treatment (24 or 48 h) and Western blotted with anti-XAB2 mAb, anti-HDAC3 polyclonal antibody, or anti-RAR α polyclonal antibody. **B**, 10 μ g of nuclear extracts of HL60 cells were Western blotted with anti-XAB2 mAb, anti-HDAC3 polyclonal antibody, or antitubulin mAb in the presence or absence of ATRA treatment (24 or 48 h). **C**, HL60 cells were treated with ATRA (1 μ mol/L) or DMSO vehicle for 24 h and submitted to analysis using confocal laser microscopy as described in Materials and Methods. **D**, chromatin immunoprecipitation assay was done using HL60 cells treated with ATRA (1 μ mol/L) or DMSO vehicle for 24 h. Extracted chromatin samples were used directly in the PCR for the input control (lanes 1 and 2). Goat polyclonal IgG was used as negative control (*cIgG*; lanes 3 and 4). Antibodies for XAB2 (lanes 5 and 6), HDAC3 (lanes 7 and 8), RAR α (lanes 9 and 10), and acetylated histone H4 (*Ac-H4*; lanes 11 and 12) were used for immunoprecipitation of the endogenous proteins. PCR was done using primers for RAR β and CYP26 promoters containing RARE as described in Materials and Methods. All experiments were done at least thrice with similar results.



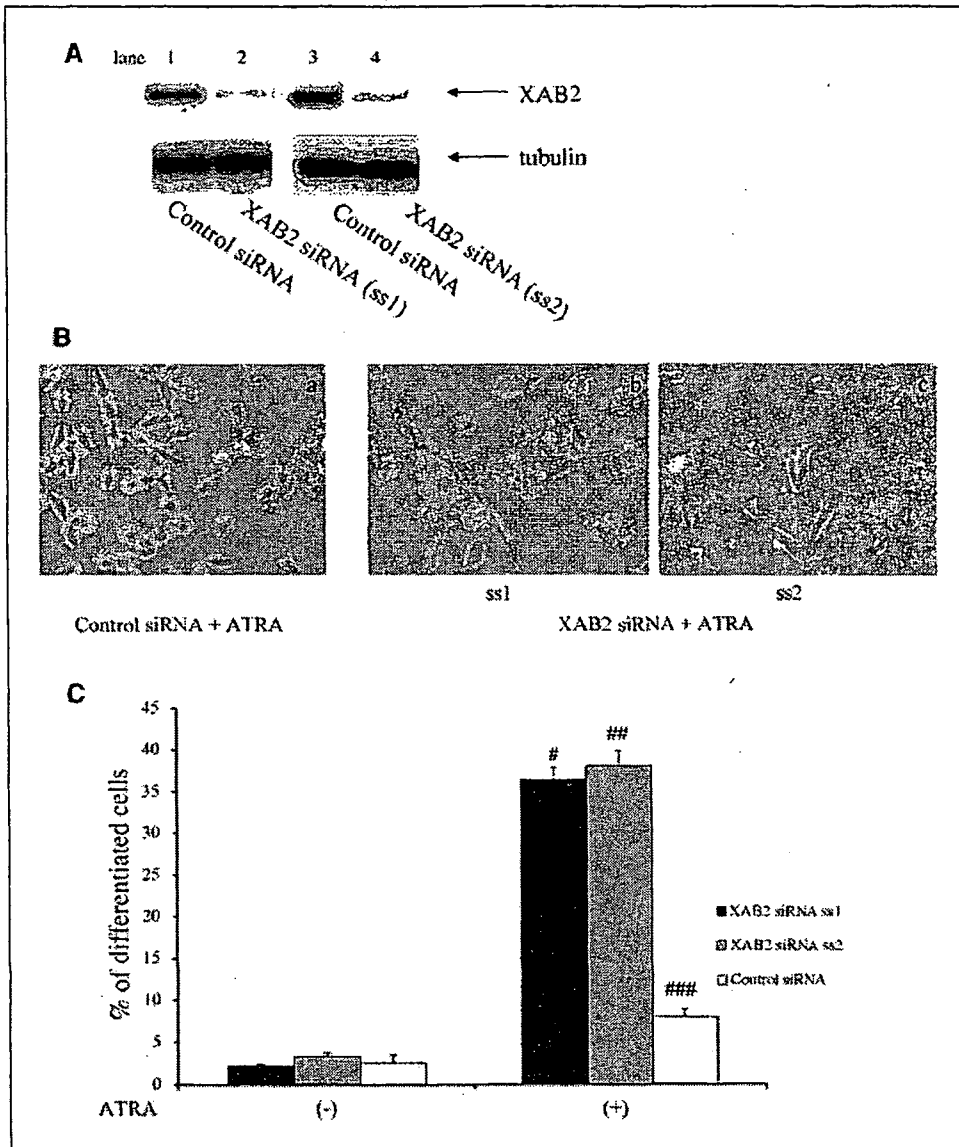


Figure 5. Knockdown of XAB2 by siRNA enhances ATRA-mediated cellular differentiation of ATRA-resistant neuroblastoma cells. **A**, ATRA-resistant neuroblastoma cell line IMR-32 was transfected with sense siRNAs of XAB2 gene (XAB2 siRNA ss1 or ss2) or with control nonsense siRNA using Nucleofector. Cell lysates were resolved by 10% SDS-PAGE and immunoblotted with anti-XAB2 mAb, followed by stripping and reprobing with antitubulin mAb. All experiments were done at least thrice with similar results. **B**, effect of siRNA against XAB2 on ATRA-induced IMR-32 cell differentiation. After 48 h of transfection with siRNA (XAB2 siRNA ss1, ss2, or control siRNA), IMR-32 cells (1×10^6 /mL) were treated with 2 μ M ATRA for an additional 24 h and photographed using phase-contrast microscopy. **C**, IMR-32 cells were treated with the same method as described in (B). Cells extending processes longer than twice the diameter of the cell body were counted as being differentiated, and two pathologists identified the morphologic changes to formation of neurite-like processes. The graph shows the percentage of differentiated cells among 1,000 quantified cells. Columns, mean of triplicate experiments; bars, SE. # and ##, significant changes compared with control ($P < 0.05$).

disengage from these genes following ATRA treatment, we did chromatin immunoprecipitation assay with HL60 cells. For this purpose, HL60 cells were treated in the presence or absence of ATRA for 24 h, followed by cross-linking by formaldehyde, and chromatin-protein complexes were immunoprecipitated using control IgG, anti-XAB2 pAb, anti-HDAC3 pAb, anti-RAR α pAb, or anti-acetyl-histone H4 pAb. Chromatin immunoprecipitation assays against endogenous RAR β and CYP26 promoters that contain RARE sequences were done (47). As shown in Fig. 4D, endogenous XAB2 and HDAC3 were recruited to RAR β and CYP26 promoters in the absence of ATRA (lanes 5 and 7). However, in the presence of ATRA, endogenous XAB2 and HDAC3 were released from RAR β and CYP26 promoters (Fig. 4D, lanes 6 and 8). On the other hand, endogenous RAR α bound to RAR β and CYP26 promoters constitutively in the presence or absence of ATRA (Fig. 4D, lanes 9 and 10). Moreover, to determine the acetylation level of histones around the RARE region of RAR β and CYP26 genes, chromatin immunoprecipitation assays with anti-acetylated histone H4 pAb were done. In the absence of ATRA, histone acetylation levels of RAR β and CYP26 promoters were decreased

(Fig. 4D, lane 11). On the other hand, in the presence of ATRA, histone acetylation level of these promoters was significantly increased (Fig. 4D, lane 12). These data suggest that XAB2 forms a complex with RAR α and HDAC3 on the same ATRA-targeted genes and that, following ATRA treatment, XAB2 and HDAC3 are disengaged from the complex, which is associated with increased level of promoter histone acetylation.

Knockdown of XAB2 by siRNA induces differentiation of ATRA-resistant neuroblastoma cell line. In view of XAB2 inhibitory effect on ATRA-mediated transcriptional activity and cellular differentiation, we examined the effect of XAB2 on ATRA-mediated cellular differentiation of ATRA-resistant cells. For this purpose, we conducted siRNA experiments using the ATRA-resistant human neuroblastoma cell line IMR-32 (53). We prepared two specific siRNAs against XAB2 as described in Materials and Methods, which effectively knocked down XAB2 expression in IMR-32 cells (Fig. 5A, top, lanes 2 and 4). Because XAB2 in IMR-32 cells was not significantly knocked down by control siRNA (Fig. 5A, top, lanes 1 and 3) and expression level of tubulin as a housekeeping protein was not affected by both siRNAs (Fig. 5A, bottom), the

observed inhibition of XAB2 expression by its siRNA was specific. In addition, the expression levels of RAR α and HDAC3 were not altered by treatment of XAB2-specific siRNA (data not shown). We next examined the effect of ATRA on cell differentiation of IMR-32 cells with XAB2 expression being knocked down by siRNA. As shown in Fig. 5B, changes consistent with cell differentiation following treatment with ATRA were not observed in ATRA-resistant IMR-32 cells treated with control siRNA (a). On the other hand, ATRA-resistant IMR-32 cells treated with two separate siRNAs against XAB2 exhibited ATRA-induced cell differentiation (b and c). Quantification of cell differentiation by ATRA in IMR-32 cells is shown in Fig. 5C. As shown in microscopic observation study (Fig. 5B, a-c), neurite-like differentiation of ATRA-resistant IMR-32 induced by ATRA treatment was significantly increased in cells treated with siRNAs against XAB2 (Fig. 5C, # and ##) compared with cell treated with control siRNA (Fig. 5C, ###). Although the precise mechanisms of ATRA resistance in IMR-32 have not yet been revealed (53), these data suggest that XAB plays an inhibitory role in ATRA-induced cell differentiation and that knockdown of XAB2 in selected ATRA-resistant tumors induces ATRA-mediated cellular differentiation.

Discussion

In this study, we showed that overexpression of XAB2 inhibited ATRA-induced cellular differentiation of the human rhabdomyosarcoma cell line MM-1-19-P and that knockdown of XAB2 by siRNA enhanced ATRA-mediated cellular differentiation of the human promyelocytic leukemia cell line HL60. Moreover, we found that XAB2 was associated with RAR α and HDAC3 in the nuclei. Finally, we showed that, following treatment with siRNA specific against XAB2, the ATRA-resistant neuroblastoma cell line IMR-32 underwent cellular differentiation when incubated with ATRA at a therapeutic concentration.

XAB2 has been isolated by virtue of its ability to interact with XPA in the yeast two-hybrid system (33). Xeroderma pigmentosum patients show striking hypersensitivity to sunlight and an extremely high incidence of skin cancer in sun-exposed areas, as well as frequently progressive neurologic degeneration (40). Antibodies against the XAB2 protein has been shown to inhibit transcription in non-UV-irradiated normal cells, strongly suggesting that XAB2 can be a novel factor involved in the transcription process itself. In addition, it has previously been shown that retinoids are effective in treating preneoplastic diseases including xeroderma pigmentosum and other dermatologic diseases such as photoaging. We thus aimed to analyze the effect of XAB2 on retinoid-mediated transcription in the present study, especially on ATRA-mediated cellular differentiation, as cancer therapy. Our *in vitro* analyses shown in Figs. 1 and 2 showed that XAB2 has an inhibitory effect on ATRA-induced cellular differentiation of ATRA-sensitive neoplastic cell lines and that ATRA-mediated transcriptional activity is inhibited by XAB2 in a dose-dependent manner. Moreover, expression levels of endogenous RAR α and HDAC3 are not affected by overexpression of exogenous XAB2 or by knockdown of XAB2 by siRNA (Figs. 2, 3, and 5). These results suggest that XAB2 plays an inhibitory role in ATRA-induced cellular differentiation by being functionally associated with the transcriptional repressor complex existing in RARE.

Our next focus in this study was to analyze the molecular mechanisms of XAB2-mediated inhibitory effect on ATRA-induced

cellular differentiation. As shown in Figs. 3 and 4, XAB2 forms a complex with RAR α and HDAC3 and is dissociated from RAR α and HDAC3 in the presence of ATRA treatment. In the absence of ligands for RAR-RXR dimers such as ATRA, genes targeted by the receptors are repressed (reviewed in ref. 3). This is due to the recruitment of HDAC-containing complexes that are tethered through corepressors, such as N-CoR/SMRT, to the nonliganded RAR-RXR dimers. Moreover, the repressive activity of the N-CoR/SMRT complex results from direct or indirect association with HDAC3, leading to the formation of a stoichiometric core complex with N-CoR/SMRT (24, 54). Our data shown in Figs. 3 and 4 suggest that XAB2 is associated with corepressor complexes that bind to RAR α and/or HDAC3 to repress transcriptional activity in the absence of ATRA. On the other hand, on binding of ligands to RAR, the corepressor interface is destabilized to generate a novel interaction surface for coactivators such as histone acetyltransferases and cAMP-responsive element binding protein-binding proteins or p300, leading to transcription initiation (3). To switch from corepressors to coactivators following ligand binding to RAR α -RXR, specific adaptors are required for dismissal and subsequent degradation by ubiquitination of corepressors N-CoR/SMRT/HDAC3 (22, 24). TBL1/TBLR1 has been reported to serve as specific adaptors for the recruitment of the ubiquitin-conjugating proteasome complex to degrade N-CoR/SMRT/HDAC3 (55). In this

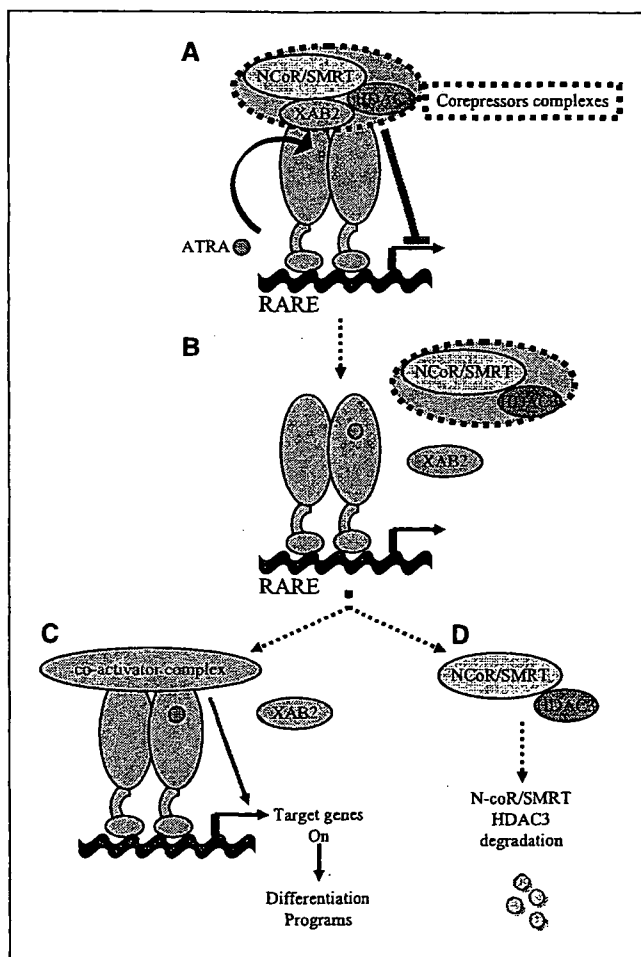


Figure 6. Model for the molecular interaction of XAB2 with RAR α /RXR and N-CoR/SMRT corepressor complexes. See text for details.

regard, although it is presently unclear whether XAB2 is degraded or recycled via an unknown pathway, XAB2 remains localized in the nuclei after ATRA treatment (Fig. 4) while still disengaging a corepressor complex containing N-CoR/SMRT, HDAC3, and XAB2 from RAR α -RXR on RARE (Fig. 4), potentially mediating corepressor/coactivator exchange.

Our findings suggest XAB2 interaction with corepressor complex containing HDAC3 and RAR α through biochemical assays revealing protein-protein association and cellular assays showing biological effects (Figs. 3 and 4). However, our data also raise questions about the functional implication of XAB2 binding to HDAC3 and RAR α . As shown in Fig. 3, endogenous XAB2 was mainly associated with HDAC3, whereas a small amount of HDAC1 was coprecipitated with XAB2. In fact, the precise mechanism of HDACs involvement in retinoid signaling is controversial (56). We therefore could not exclude the possibility that HDAC1 is involved in XAB2-associated retinoid signaling, a topic which will be elucidated in future studies. Another issue raised by our findings is whether XAB2 is involved in other nuclear receptor systems than RAR α , such as vitamin D receptor or thyroid hormone receptor (24). Indeed, corepressor complex such as N-CoR/SMRT/HDACs also regulates other nuclear receptor systems than RAR α (19), suggesting that XAB2 may be possibly involved in other transcriptional systems. Whereas data shown in our present study were obtained in transient transfection systems, we plan to examine the inhibitory effect of XAB2 on retinoid signaling or other nuclear receptor systems with stably transfected systems of overexpression and siRNAs in future studies.

On the basis of our results, we propose a model to describe the role of XAB2 in cell responsiveness to retinoids (Fig. 6). In this model, unliganded RAR α -RXR heterodimers bind to RARE and recruit HDAC3 complexes containing XAB2, followed by suppression of target genes for differentiation blockade (Fig. 6, a). On

binding of ligands such as ATRA to RAR α , N-CoR/SMRT/HDAC3 corepressors are dismissed and subsequently degraded by ubiquitination, which is mediated by specific adaptors such as TBL1/TBLR1 (Fig. 6, b and c). Subsequently, coactivator complexes such as cAMP-responsive element binding protein-binding protein or p300 are recruited, leading to target gene activation and differentiation programs (Fig. 6, d). The potential clinical relevance of this model is revealed by the fact that pharmacologic concentrations of ATRA are required in the presence of XAB2 to disrupt this complex. However, combining ATRA treatment with knockdown of XAB2 by siRNA allows for the dissociation of the repressor complex containing HDAC3 and XAB2, leading subsequently to cellular differentiation.

Work over the past decades has shown that retinoids and their cognate receptors are required for the proper functioning of a number of organs, including the skin, and of the neuronal system and for prevention and treatment of selected cancers (17, 57, 58). One of the important objectives of cancer differentiation therapy is to selectively induce growth inhibition through RAR-dependent pathways. Our findings therefore should have broad implications for potential therapy not only for ATRA-sensitive cancer such as acute promyelocytic leukemia and rhabdomyosarcoma but also for ATRA-resistant solid tumors including neuroblastoma.

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References

- Lotan R. Retinoids in cancer chemoprevention. *FASEB J* 1996;10:1031-9.
- Freemantle SJ, Spinella MJ, Dmitrovsky E. Retinoids in cancer therapy and chemoprevention: promise meets resistance. *Oncogene* 2003;22:7305-15.
- Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. *Nat Rev Cancer* 2001;1:181-93.
- Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. *Science* 1997;278:1073-7.
- Sun SY, Lotan R. Retinoids and their receptors in cancer development and chemoprevention. *Crit Rev Oncol Hematol* 2002;41:41-55.
- Thacher SM, Vasudevan J, Chandraratna RA. Therapeutic applications for ligands of retinoid receptors. *Curr Pharm Des* 2000;6:25-58.
- Huang ME, Ye YC, Chen SR, et al. Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood* 1988;72:567-72.
- Tallman MS, Andersen JW, Schiffer CA, et al. All-trans-retinoic acid in acute promyelocytic leukemia. *N Engl J Med* 1997;337:1021-8.
- de Thé H, Chomienne C, Lanotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukaemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature* 1990;347:558-61.
- Kakizuka A, Miller WH, Jr., Umesono K, et al. Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcription factor. *PML Cell* 1991;66:663-74.
- Piazza F, Gurrieri C, Pandolfi PP. The theory of APL. *Oncogene* 2001;20:7216-22.
- Ferrara FF, Fazi F, Bianchini A, et al. Histone deacetylase-targeted treatment restores retinoic acid signaling and differentiation in acute myeloid leukemia. *Cancer Res* 2001;61:2-7.
- Grignani F, De Matteis S, Nervi C, et al. Fusion proteins of the retinoic acid receptor- α recruit histone deacetylase in promyelocytic leukaemia. *Nature* 1998;391:815-8.
- Lin RJ, Nagy L, Inoue S, Shao W, Miller WH, Jr., Evans RM. Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature* 1998;391:811-4.
- Warrell RP, Jr., He LZ, Richon V, Calleja E, Pandolfi PP. Therapeutic targeting of transcription in acute promyelocytic leukemia by use of an inhibitor of histone deacetylase. *J Natl Cancer Inst* 1998;90:1621-5.
- Leid M, Kastner P, Lyons R, et al. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 1992;68:377-95.
- Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB J* 1996;10:940-54.
- Urnov FD, Wolffe AP, Guschin D. Molecular mechanisms of corepressor function. *Curr Top Microbiol Immunol* 2001;254:1-33.
- Chen JD, Evans RM. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 1995;377:454-7.
- Chen JD, Umesono K, Evans RM. SMRT isoforms mediate repression and anti-repression of nuclear receptor heterodimers. *Proc Natl Acad Sci U S A* 1996;93:7567-71.
- Xu L, Glass CK, Rosenfeld MG. Coactivator and corepressor complexes in nuclear receptor function. *Curr Opin Genet Dev* 1999;9:140-7.
- Guenther MG, Barak O, Lazar MA. The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. *Mol Cell Biol* 2001;21:6091-101.
- Wen YD, Perissi V, Staszewski LM, et al. The histone deacetylase-3 complex contains nuclear receptor corepressors. *Proc Natl Acad Sci U S A* 2000;97:7202-7.
- Li J, Wang J, Wang J, et al. Both corepressor proteins SMRT and N-CoR exist in large protein complexes containing HDAC3. *EMBO J* 2000;19:4342-50.
- Zhang J, Kalkum M, Chait BT, Roeder RG. The N-CoR-HDAC3 nuclear receptor corepressor complex inhibits the JNK pathway through the integral subunit GPS2. *Mol Cell* 2002;9:611-23.
- Sternsdorf T, Phan VT, Maunakea ML, et al. Forced retinoic acid receptor α homodimers prime mice for APL-like leukemia. *Cancer Cell* 2006;9:81-94.
- Kwok C, Zeisig BB, Dong S, So CW. Forced homodimerization of RAR α leads to transformation of primary hematopoietic cells. *Cancer Cell* 2006;9:95-108.
- Coffey DC, Kutko MC, Glick RD, et al. Histone deacetylase inhibitors and retinoic acids inhibit growth of human neuroblastoma *in vitro*. *Med Pediatr Oncol* 2000;35:577-81.
- Gottlicher M, Minucci S, Zhu P, et al. Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 2001;20:6969-78.
- Marks PA, Richon VM, Rifkin RA. Histone deacetylase inhibitors: inducers of differentiation or apoptosis of transformed cells. *J Natl Cancer Inst* 2000;92:1210-6.
- Petti MC, Fazi F, Gentile M, et al. Complete remission through blast cell differentiation in PLZF/RAR α -positive acute promyelocytic leukemia: *in vitro* and *in vivo* studies. *Blood* 2002;100:1065-7.
- Zhou DC, Kim SH, Ding W, Schultz C, Warrell RP, Jr., Gallagher RE. Frequent mutations in the ligand-binding domain of PML-RAR α after multiple relapses of acute

- promyelocytic leukemia: analysis for functional relationship to response to all-*trans* retinoic acid and histone deacetylase inhibitors *in vitro* and *in vivo*. *Blood* 2000;99:1356-63.
33. Nakatsu Y, Asahina H, Citterio E, et al. XAB2, a novel tetratricopeptide repeat protein involved in transcription-coupled DNA repair and transcription. *J Biol Chem* 2000;275:34931-7.
 34. Yonemasu R, Minami M, Nakatsu Y, et al. Disruption of mouse XAB2 gene involved in pre-mRNA splicing, transcription and transcription-coupled DNA repair results in preimplantation lethality. *DNA Repair (Amst)* 2005;4:479-91.
 35. Fouteri M, Vermeulen W, van Zeeland AA, Mullenders LH. Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II *in vivo*. *Mol Cell* 2006;23:471-82.
 36. Hoeijmakers JH. Genome maintenance mechanisms for preventing cancer. *Nature* 2001;411:366-74.
 37. Lindahl T, Wood RD. Quality control by DNA repair. *Science* 1999;286:1897-905.
 38. Berneburg M, Lehmann AR. Xeroderma pigmentosum and related disorders: defects in DNA repair and transcription. *Adv Genet* 2001;43:71-102.
 39. Daya-Grosjean L, Sarasin A. The role of UV induced lesions in skin carcinogenesis: an overview of oncogene and tumor suppressor gene modifications in xeroderma pigmentosum skin tumors. *Mutat Res* 2005;571:43-56.
 40. Lambert WC. Xeroderma pigmentosum. *Neth J Med* 1989;86:383-6.
 41. Lehmann AR, Bridges BA. Sunlight-induced cancer: some new aspects and implications of the xeroderma pigmentosum model. *Br J Dermatol* 1990;122 Suppl 35: 115-9.
 42. Ishii T, Ohnuma K, Murakami A, et al. SS-A/Ro52, an autoantigen involved in CD28-mediated IL-2 production. *J Immunol* 2003;170:3653-61.
 43. Sandquist D, Williams TH, Sahu SK, Kataoka S. Morphological differentiation of a murine neuroblastoma clone in monolayer culture induced by dexamethasone. *Exp Cell Res* 1978;113:375-81.
 44. Ohnuma K, Munakata Y, Ishii T, et al. Soluble CD26/dipeptidyl peptidase IV induces T cell proliferation through CD86 up-regulation on APCs. *J Immunol* 2001; 167:6745-55.
 45. Ohnuma K, Yamochi T, Uchiyama M, et al. CD26 up-regulates expression of CD86 on antigen-presenting cells by means of caveolin-1. *Proc Natl Acad Sci U S A* 2004; 101:14186-91.
 46. Hosoi H, Sugimoto T, Hayashi Y, et al. Differential expression of myogenic regulatory genes, MyoD1 and myogenin, in human rhabdomyosarcoma sublines. *Int J Cancer* 1992;50:977-83.
 47. Bastien J, Rochette-Egly C. Nuclear retinoid receptors and the transcription of retinoid-target genes. *Gene* 2004;328:1-16.
 48. Drayson MT, Michell RH, Durham J, Brown G. Cell proliferation and CD11b expression are controlled independently during HL60 cell differentiation initiated by 1,25- α -dihydroxyvitamin D₃ or all-*trans*-retinoic acid. *Exp Cell Res* 2001;266:126-34.
 49. Collins SJ. The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression. *Blood* 1987;70:1233-44.
 50. Minucci S, Nervi C, Lo Coco F, Pelicci PG. Histone deacetylases: a common molecular target for differentiation treatment of acute myeloid leukemias? *Oncogene* 2001;20:3110-5.
 51. Lovat PE, Annicchiarico-Petruzzelli M, Corazzari M, et al. Differential effects of retinoic acid isomers on the expression of nuclear receptor co-regulators in neuroblastoma. *FEBS Lett* 1999;445:415-9.
 52. Reynolds CP, Wang Y, Melton LJ, Einhorn PA, Slamon DJ, Maurer BJ. Retinoic-acid-resistant neuroblastoma cell lines show altered MYC regulation and high sensitivity to fenretinide. *Med Pediatr Oncol* 2000;35: 597-602.
 53. Joshi S, Guleria R, Pan J, DiPette D, Singh US. Retinoic acid receptors and tissue-transglutaminase mediate short-term effect of retinoic acid on migration and invasion of neuroblastoma SH-SY5Y cells. *Oncogene* 2006;25:240-7.
 54. Codina A, Love JD, Li Y, Lazar MA, Neuhaus D, Schwabe JW. Structural insights into the interaction and activation of histone deacetylase 3 by nuclear receptor corepressors. *Proc Natl Acad Sci U S A* 2005; 102:6009-14.
 55. Perissi V, Aggarwal A, Glass CK, Rose DW, Rosenfeld MG. A corepressor/coactivator exchange complex required for transcriptional activation by nuclear receptors and other regulated transcription factors. *Cell* 2004; 116:511-26.
 56. Rosenfeld MG, Lunyak VV, Glass CK. Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 2006;20:1405-28.
 57. Corcoran J, Maden M. Nerve growth factor acts via retinoic acid synthesis to stimulate neurite outgrowth. *Nat Neurosci* 1999;2:307-8.
 58. DiGiovanna JJ. Retinoid chemoprevention in patients at high risk for skin cancer. *Med Pediatr Oncol* 2001;36: 564-7.

ORIGINAL ARTICLE

Crk-associated substrate lymphocyte type regulates transforming growth factor- β signaling by inhibiting Smad6 and Smad7

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Crk-associated substrate lymphocyte type (Cas-L) is a 105 kDa docking protein with diverse functional properties, including regulation of cell division, proliferation, migration and adhesion. Cas-L is also involved in β 1 integrin- or antigen receptor-mediated signaling in B and T cells. In the present study, we demonstrate that Cas-L potentiates transforming growth factor- β (TGF- β) signaling pathway by interacting with Smad6 and Smad7. Immunoprecipitation experiments reveal that single domain deletion of full-length Cas-L completely abolishes its docking function with Smad6 and Smad7, suggesting that the natural structure of Cas-L is necessary for its association with Smad6 and Smad7. On the other hand, both N-terminal and C-terminal deletion mutants of Smad6 and Smad7 still retain their docking ability to Cas-L, suggesting that Smad6 and Smad7 possess several binding motifs to Cas-L. Moreover, Cas-L interaction with Mad-homology (MH)2 domain, but not with MH1 domain of Smad6 or Smad7, ameliorates TGF- β -induced signaling pathway. Finally, depletion of Cas-L by small-interfering RNA oligo attenuates TGF- β -induced growth inhibition of Huh-7 cells, with a concomitant reduction in phosphorylation of Smad2 and Smad3. These results strongly suggest that Cas-L is a potential regulator of TGF- β signaling pathway.

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Keywords: Cas-L; Smad6; Smad7; TGF- β ; cancer

Introduction

β 1 integrins exhibit a variety of biological functions through specific interaction with their ligands (Hynes, 1992; Juliano and Haskill, 1993). We previously

demonstrated that the 105 kDa protein (pp105) termed Cas-L (Cas lymphocyte type) (Minegishi *et al.*, 1996) or human enhancer of filamentation 1 (HEF1) (Law *et al.*, 1996) is a focal adhesion kinase (FAK)-associated docking protein that is heavily tyrosine phosphorylated by FAK and Src family kinases upon engagement with β 1 integrin and the T-cell antigen receptor complex in T cells (Minegishi *et al.*, 1996; Tachibana *et al.*, 1997; Ohashi *et al.*, 1998). Transfection of Cas-L into Jurkat cells markedly enhanced cell motility (Ohashi *et al.*, 1999) and interleukin-2 production (Kamiguchi *et al.*, 1999) following β 1 integrin engagement. These results clearly indicate that Cas-L plays a key role in β 1 integrin-mediated signal transduction and cell migration.

The transforming growth factor- β (TGF- β) superfamily of cytokines has diverse functional roles during embryonic development and tissue homeostasis (Derynck and Zhang, 2003). Disruption of TGF- β signaling is responsible for a number of developmental disorders (Datta and Moses, 2000), fibrotic diseases (Yagil *et al.*, 2005) and human cancers (Kato *et al.*, 2002). Although TGF- β acts as a tumor suppressor (Kato *et al.*, 2002), it also plays opposite roles in tumor progression. As carcinogenesis proceeds and tumor cells acquire resistance to TGF- β -induced growth arrest, TGF- β accelerates cancer cell progression. Therefore, it is quite important to control the sensitivity to TGF- β for regulation of growth of cancer cells. The TGF- β superfamily transmits signals via cell surface complexes of type I and type II receptor serine/threonine kinase and intracellular Smad transcription factors, which are classified into three functional groups (Derynck and Zhang, 2003). Receptor-regulated Smads and R-Smads (Smad1, 2, 3, 5 and 8) are phosphorylated by type I receptor and oligomerize with the common mediator, Co-Smad (Smad4) (Grönroos *et al.*, 2002; Sugano *et al.*, 2003). These heteromeric complexes are translocated into the nucleus to regulate gene transcription by either association with DNA-binding proteins or direct binding to promoter sequences in target genes (Sugano *et al.*, 2003). On the other hand, inhibitory-Smads, I-Smads (Smads6 and 7) are negative signal regulators that block R-Smad phosphorylation by the type I receptor, concomitantly inducing type I receptor dephosphorylation

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and proteasomal degradation (Heldin *et al.*, 1997; Nakao *et al.*, 1997; Chacko *et al.*, 2004).

We recently demonstrated that Cas-L is upregulated in human T-lymphotropic virus type I (HTLV-I) Tax transgenic mice (Miyake-Nishijima *et al.*, 2003), and that Cas-L regulates Tax-nuclear factor- κ B pathway by direct interaction with Tax (Iwata *et al.*, 2005). Cas-L was previously reported to be involved in TGF- β signaling pathway. For example, under certain circumstances, TGF- β induces gene transcription and protein expression of Cas-L with no effect on the stability of either Cas-L protein or mRNA (Fashena *et al.*, 2002), whereas in other experimental conditions, TGF- β promotes proteasomal degradation of Cas-L (Zhang *et al.*, 2000). However, the biological significances of Cas-L in TGF- β signaling remain to be clarified. Our search for putative Cas-L associated proteins to further investigate the biological role of Cas-L led to the identification of Smad6 and Smad7, the negative signal regulators of TGF- β signaling pathways. In this study, we determined the functional significance of Cas-L interaction with I-Smads, Smad6 and Smad7. We found that Cas-L facilitates TGF- β signaling, and that the mechanism responsible for this effect appears to be the abrogation of recruitment of I-Smads to TGF- β type I receptor induced by the Cas-L-I-Smads interaction, subsequently, resulting in the acceleration of activated R-Smads by TGF- β . On the basis of our findings, we propose that the Cas-L can confer sensitivity to TGF- β , with implications for cell development, immune response, inflammatory disease and tumor biology.

Results

Cas-L interacts with Smad6 and Smad7

To understand the biological significance of Cas-L, we performed yeast two-hybrid cDNA cloning in search for Cas-L binding proteins as described previously (Iwata *et al.*, 2005). We screened over 1×10^6 independent clones from the cDNA library of the lymphoblastic cell line SLB-I. Among 180 clones selected by histidine auxotrophy and β -galactosidase (β -gal) expression, we identified a clone containing Smad7 cDNA (aa 203–353) in frame with GAL4 activator domain.

We next examined the association of exogenous Cas-L protein with Smad6 and Smad7. Unlike the results from the yeast two-hybrid assay using SLB-I cDNA library, co-transfection study with 293 T cells using flag-tagged Smad6 or Smad7 with c-myc-tagged

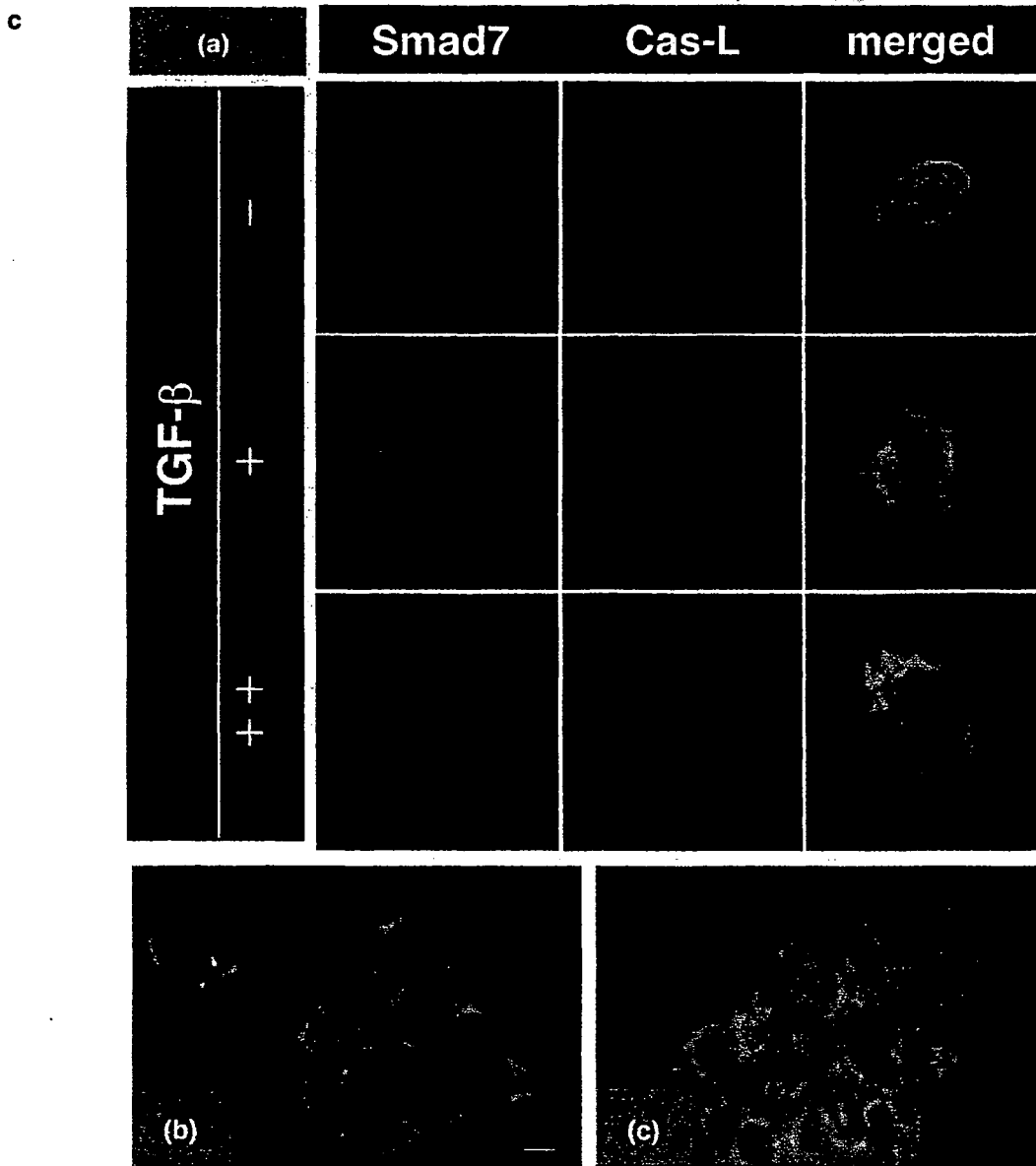
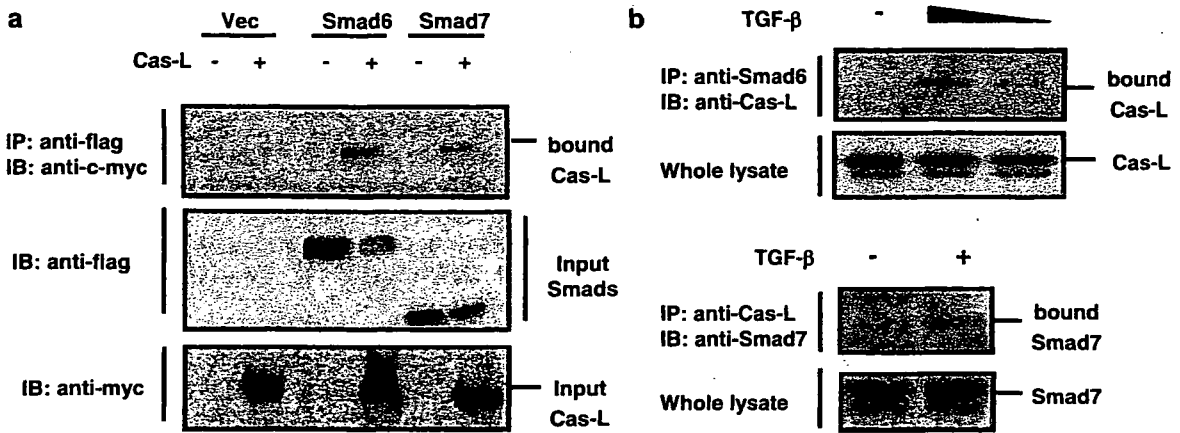
Cas-L revealed that Cas-L specifically immunoprecipitated with both Smad6 and Smad7 before TGF- β treatment (Figure 1a). This discrepancy arose from the cell-type specific differentially expressed transcripts (Supplementary Figure 1). Moreover, immunoprecipitation study of endogenous proteins revealed that the amount of Cas-L binding to Smad6 was increased with increasing doses of TGF- β (Figure 1b, upper panels). Similarly, endogenous Smad7–Cas-L binding was accelerated by TGF- β treatment (Figure 1b, lower panels).

To further investigate the intracellular events associated with the interaction of these proteins after TGF- β treatment, we next examined colocalization of Cas-L and I-Smads using confocal microscopy. Co-transfection study of flag-tagged Smad7 with c-myc-tagged Cas-L showed that Smad7 and Cas-L were dynamically colocalized in the cytoplasm, but not in the nucleus, following TGF- β treatment, with Smad7 gradually localizing in the cytoplasm (Figure 1c). Colocalization of Smad6 and Cas-L was also upregulated by TGF- β treatment (data not shown). These results thus suggested that Cas-L associates with both Smad6 and Smad7, with the association between Cas-L and both Smad6 and Smad7 being accelerated by TGF- β treatment.

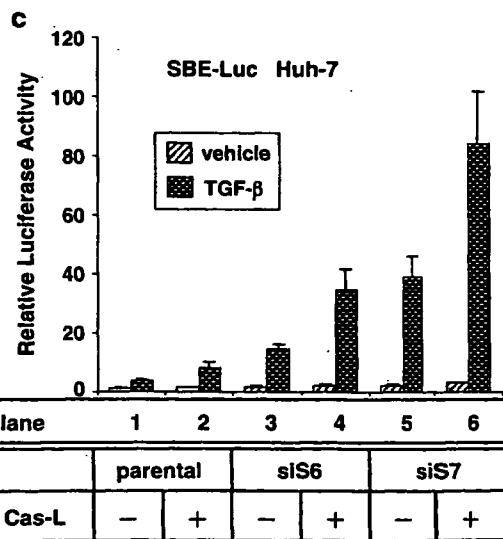
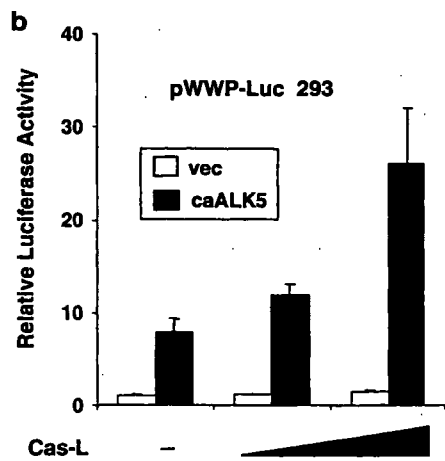
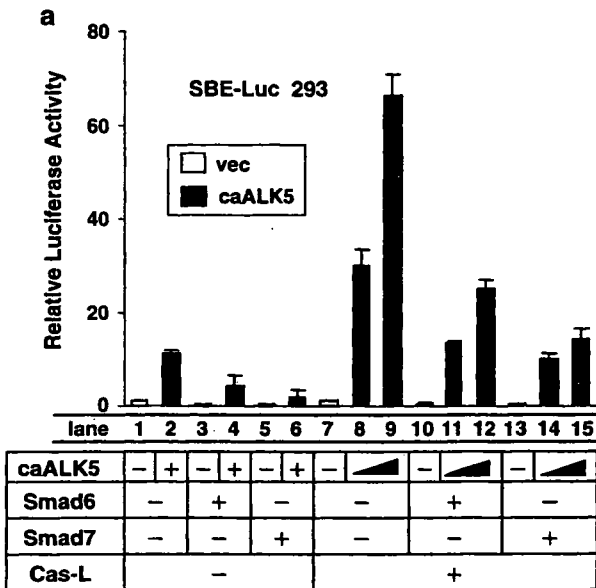
Cas-L counteracts I-Smads-induced inhibition of transcriptional activity by TGF- β

As Cas-L interacted with Smad6 and Smad7, we further examined the functional consequences of Cas-L on TGF- β -induced transcriptional activity using reporter assay. As seen in Figure 2a, lane 7, Cas-L merely upregulated the basal (endogenous) transcriptional activity. Importantly, constitutively active activin-receptor-like kinase-5 (ALK5) (a mimicry of TGF- β) exaggerated the Cas-L-induced upregulation of transcriptional activity in a dose-dependent manner, suggesting that Cas-L induced TGF- β -mediated transcriptional activation (Figure 2a, lanes 8 and 9). Smad7 is known to be more potent than Smad6 in inhibiting TGF- β , whereas Smad6 is more potent than Smad7 to another TGF- β superfamily bone morphogenetic protein (Derynck *et al.*, 2001). As seen in Figure 2a, lanes 3–6, Smad7 inhibited transcriptional activity more strongly than Smad6, with constitutively active ALK5 having a markedly enhancing effect. Importantly, Cas-L counteracted the transcriptional inhibition by Smad6 (Figure 2a, lanes 3 and 4 vs lanes 10 and 11) and Smad7 (Figure 2a, lanes 5 and 6 vs lanes 13 and 14). In addition, this effect was strengthened by increasing doses of constitutively active ALK5 (Figure 2a, lane 11

Figure 1 Cas-L interacts with Smad6 and Smad7. (a) C-myc-tagged full-length Cas-L and flag-tagged Smad6 or Smad7 were co-transfected in 293 T cells, followed by immunoprecipitation using anti-flag antibody, subjected to immunoblotting. Upper panel indicates Cas-L precipitates with flag-Smad6 or flag-Smad7, but not with control vector. (b) Huh-7 cells were treated by TGF- β , subsequently subjected to immunoprecipitation by anti-Smad6, or anti-Cas-L, then followed by SDS-PAGE and immunoblotting. Detection of immunoprecipitated proteins were by anti-Smad6 or anti-Cas-L. (c) C-myc-tagged full-length Cas-L and flag-tagged Smad7 were co-transfected in 293 T cells, followed by immunocytochemistry using anti-flag-FITC antibody and anti-c-myc-Cy3 antibody. (a) Dose-dependent effect of TGF- β on Cas-L-I-Smads interaction was assessed by confocal microscope. (b) Merged view of Cas-L (red) and I-Smads (green) with low-dose TGF- β in lower magnification. (c) Merged view of Cas-L (red) and I-Smads (green) with high-dose TGF- β in lower magnification. Scale bar indicates 5 μ m.



vs lane 12 and lane 14 vs lane 15). These results suggested that Cas-L counteracts the transcriptional inhibition by I-Smads, and this effect becomes more potent in the presence of ligand stimulation.



Cyclin-dependent kinase inhibitors, including p21^{cip1/waf1}, are known to be the downstream signaling molecules of TGF- β signaling pathway. A reporter plasmid of p21^{cip1/waf1}, pWWP-Luc, was employed to evaluate whether Cas-L could perturb the transcriptional activity of p21^{cip1/waf1}. As seen in Figure 2b, Cas-L profoundly enhanced pWWP-Luc transcriptional activity in 293 cells compared to the control in a dose-dependent manner, suggesting that abundance of Cas-L is the determinant of transcriptional activation by TGF- β .

Using Huh-7 cells to confirm the results seen in 293 cells, transcriptional activity of SBE-Luc were upregulated with Cas-L co-transfection (Figure 2c, lanes 1 and 2). As expected, small-interfering RNA (siRNA) of both I-Smads induced transcriptional activation by TGF- β (Figure 2c, lanes 3 and 5). It should be noted that the combination of siRNA of Smad6 or Smad7 and Cas-L had an additive or accumulative effect on TGF- β -induced transcriptional upregulation (Figure 2c, lanes 4 and 6). These results suggested that Cas-L upregulates transcriptional activity induced by TGF- β by interfering with I-Smads.

Depletion of endogenous Cas-L by siRNA in Huh-7 cells results in resistance against TGF- β -induced growth inhibition

As overexpression of Cas-L enhanced sensitivity to TGF- β signaling, we examined the effect of Cas-L siRNA oligo-induced depletion of Cas-L on TGF- β signaling. Activation of Smad2 and Smad3 in parental Huh-7 cells occurred from 15 min to 6 h following TGF- β treatment, peaking at 30 min (Figure 3a). Introduction of siCas-L inhibited TGF- β -induced activation of Smad2 and Smad3, with control oligo having no effect (Figure 3b). Expanding on these results, effect of siCas-L on Huh-7 proliferation was assessed. Growth of parental Huh-7 cells was specifically inhibited by TGF- β in a dose-dependent manner (Figure 3c), whereas siCas-L-transfected Huh-7 cells became much less sensitive to TGF- β -induced growth arrest (Figure 3d). These results suggested that Cas-L plays a role in TGF- β signaling pathway, possibly regulating cellular sensitivity to TGF- β .

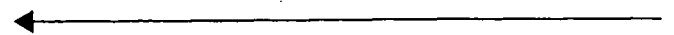


Figure 2 Cas-L upregulates TGF- β -induced transcriptional activity. Each reporter plasmid was co-transfected with renilla and firefly reporter plasmids as described in Materials and methods. Results with renilla reporter plasmid were used for the normalization of transfection efficiency. (a) In 293 cells, SBE-Luc reporter plasmid was transfected with pcDEF6 vector, Smad6 or Smad7 in the presence or absence of constitutive active ALK5. (b) In 293 cells, pWWP-Luc reporter plasmid was transfected with pEB6 vector, 0.1 μ g Cas-L or 0.2 μ g Cas-L in the presence or absence of constitutive active ALK5. (c) In Huh-7 cells, or Huh-7 cells with endogenous Smad6 or Smad7 depleted, SBE-Luc reporter plasmid was transfected with pEB6 vector, or Cas-L. At 48 h of transfection, all cells were treated with 1 ng/ml TGF- β . Depletion of endogenous I-Smads was performed as described in Materials and methods.