T cells (Tregs) [32] and to induce hematopoiesis and acute phase inflammation [15, 32], partially by blocking signaling that facilitates Tregs expansion [5]. Accordingly, our results suggest that both TARC and RANTES in combination with GM-CSF may interfere with the suppressive effects of Tregs, by enhancing IL-6 secretion and creating a microenvironment that does not support tumor growth. However, it is still possible that TARC attracts CCR4+ Treg cells [18]. Our immunohistochemical analysis showing less FoxP3$^+$ positive cells in tumors from mice treated with both irW/GM + rmTARC and irW/GM + rmRANTES further suggests that IL-6 is necessary to suppress Tregs expansion.

We have demonstrated the vaccine efficacy of GM-CSF-transduced tumor cells (auto-GVAX) in preclinical and clinical settings. Particularly, two of four patients had long-term survival greater 5 years with low dose IL-2 and one patient survived more than 8 years in PS0. The combination of auto-GVAX with other immune therapies is thought to potentiate the tumor specific immunity of auto-GVAX. The combination of GVAX with a neutralizing antibody to cytotoxic T lymphocyte antigen-4 (CTLA-4) is one of the strong candidates [6]. In this study, we demonstrated that TARC and RANTES enhanced antitumor immunity induced by GM-CSF, most likely by augmenting dominant Th2 and Th1 T cell immune responses, respectively, in mouse WEHI3B tumor models. These results also suggest that these chemokines have a clinical application in tumor vaccination. Namely, GVAX vaccination with the addition of either recombinant RANTES and/or TARC expression vectors is a promising second-generation GVAX gene therapy candidate. This latter novel gene therapy is currently under investigation using several viral vectors. In this regimen, GVAX may effectively enhance immune recognition of tumor antigens via DC activation and subsequent migration of activated T cells into the tumor tissue, leading to tumor eradication. Further preclinical investigations using different malignant cells types are required to prove this hypothesis.

Acknowledgments We thank Dr. Shinji Okano and Dr. Yoshikazu Yonemitsu, Kyushu University, for their helpful advices. We also thank Ms. Michiyo Okada for excellent technical assistance. This work was supported by grants from the Ministry of Health, Labor, and Welfare and the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

1. Brunner C, Seiderer J, Schlamp A, Bidlingmaier M, Eigler A, Haimerl W, Lehr HA, Krieg AM, Hartmann G, Endres S (2000) Enhanced dendritic cell maturation by TNF-alpha or cytidine-phosphate-guanosine DNA drives T cell activation in vitro and therapeutic anti-tumor immune responses in vivo. J Immunol 165:6278
2. Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J (1992) GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. Nature 360:258
3. Chu Y, Xia M, Lin Y, Li A, Wang Y, Liu R, Xiong S (2006) Th2-dominated antitumor immunity induced by DNA immunization with the genes coding for a basal core peptide PDTRP and GM-CSF. Cancer Gene Ther 13:510
4. Costello R, O'Callaghan T, Sebahoun G (2005) [Eosinophils and antitumour response]. Rev Med Interne 26:479
5. Doganci A, Eigenbrod T, Krug N, De Sanctis GT, Hausding M, Erpenbeck VJ, Haddad el B, Lehr HA, Schmitt E, Bopp T, Kallen KJ, Herz U, Schmitt S, Luft C, Hecht O, Hohlfeld JM, Ito H, Nishimoto N, Yoshizaki K, Kishimoto T, Rose-John S, Renz H, Neurath MF, Galle PR, Finotto S (2005) The IL-6R alpha chain controls lung CD4+CD25+ Treg development and function during allergic airway inflammation in vivo. J Clin Invest 115:313
6. Dranoff G (2005) CTLA-4 blockade: unveiling immune regulation. J Clin Oncol 23:662
7. Dranoff G, Jaffee E, Lazenby A, Golumbek P, Levitsky H, Brose K, Jackson V, Hamada H, Pardoll D, Mulligan RC (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. Proc Natl Acad Sci USA 90:3539
8. Ellem KA, O'Rourke MG, Johnson GR, Parry G, Misko IS, Schmidt CW, Parsons PG, Burrows SR, Cross S, Fell A, Li CL, Bell JR, Dubois PJ, Moss DJ, Good MF, Kelso A, Cohen LK, Dranoff G, Mulligan RC (1997) A case report: immune responses and clinical course of the first human use of granulocyte/macrophage-colony-stimulating-factor-transduced autologous melanoma cells for immunotherapy. Cancer Immunol Immunother 44:10
9. Elsner J, Escher SE, Forssmann U (2004) Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. Allergy 59:1243
10. Fujii S, Hamada H, Fujimoto K, Shimomura T, Kawakita M (1999) Activated dendritic cells from bone marrow cells of mice receiving cytokine-expressing tumor cells are associated with the enhanced survival of mice bearing syngeneic tumors. Blood 93:4328
11. Granucci F, Girolomoni G, Lutz MB, Ricciardi-Castagnoli P (1995) Recombinant GM-CSF induces cytokine production in mouse dendritic cell clones. Adv Exp Med Biol 378:31
12. Hege KM, Carbone DP (2003) Lung cancer vaccines and gene therapy. Lung Cancer 41(Suppl 1):S103
13. Hege KM, Jooss K, Pardoll D (2006) GM-CSF gene-modifed cancer cell immunotherapies: of mice and men. Int Rev Immunol 25:321
14. Heufler C, Koch F, Schuler G (1988) Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. J Exp Med 167:700
15. Hirano T (1998) Interleukin 6 and its receptor: ten years later. Int Rev Immunol 16:249
16. Imai T, Nagira M, Takagi S, Kakizaki M, Nishimura M, Wang J, Gray PW, Matsushima K, Yoshie O (1999) Selective recruitment of CCR4-bearing Th2 cells toward antigen-presenting cells by the CC chemokines thymus and activation-regulated chemokine and macrophage-derived chemokine. Int Immunol 11:81
17. Inaba K, Inaba M, Romani N, Aya H, Deguchi M, Ikehara S, Muramatsu S, Steinman RM (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med 176:1693
18. Ishida T, Ueda R (2006) CCR4 as a novel molecular target for immunotherapy of cancer. Cancer Sci 97:1139

19. Jaffee EM, Hruban RH, Biedrzycki B, Laheru D, Schepers K, Sauter PR, Goemann M, Coleman J, Grochow L, Donehower RC, Lillemoe KD, O'Reilly S, Abrams RA, Pardoll DM, Cameron JL, Yeo CJ (2001) Novel allogeneic granulocyte-macrophage colony-stimulating factor-secreting tumor vaccine for pancreatic cancer: a phase I trial of safety and immune activation. J Clin Oncol 19:145
20. Kato Y, Pawankar R, Kimura Y, Kawana S (2006) Increased expression of RANTES, CCR3 and CCR5 in the lesional skin of patients with atopic eczema. Int Arch Allergy Immunol 139:245
21. Kinoshita Y, Kono T, Yasumoto R, Kishimoto T, Wang CY, Haas GP, Nishisaka N (2001) Antitumor effect on murine renal cell carcinoma by autologous tumor vaccines genetically modified with granulocyte-macrophage colony-stimulating factor and interleukin-6 cells. J Immunother 24:205
22. Levitsky HI, Montgomery J, Ahmadzadeh M, Staveley-O'Carroll K, Guarnieri F, Longo DL, Kwak LW (1996) Immunization with granulocyte-macrophage colony-stimulating factor-transduced, but not B7-1-transduced, lymphoma cells primes idiotype-specific T cells and generates potent systemic antitumor immunity. J Immunol 156:3858
23. Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B (1996) Activation of NK cells by CC chemokines. Chemotaxis, Ca2+ mobilization, and enzyme release. J Immunol 156:322
24. Loetscher P, Uguccioni M, Bordoli L, Baggiolini M, Moser B, Chizzolini C, Dayer JM (1998) CCR5 is characteristic of Th1 lymphocytes. Nature 391:344
25. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25:677
26. Miller G, Pillarisetty VG, Shah AB, Lahrs S, Xing Z, DeMatteo RP (2002) Endogenous granulocyte-macrophage colony-stimulating factor overexpression in vivo results in the long-term recruitment of a distinct dendritic cell population with enhanced immunostimulatory function. J Immunol 169:2875
27. Murphy PM (2002) International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. Pharmacol Rev 54:227
28. Nagai E, Ogawa T, Kielian T, Ikubo A, Suzuki T (1998) Irradiated tumor cells adenovirally engineered to secrete granulocyte/macrophage-colony-stimulating factor establish antitumor immunity and eliminate pre-existing tumors in syngeneic mice. Cancer Immunol Immunother 47:72
29. Nakazaki Y, Hase H, Inoue H, Beppu Y, Meng XK, Sakaguchi G, Kurita R, Asano S, Nakamura Y, Tani K (2006) Serial analysis of gene expression in progressing and regressing mouse tumors implicates the involvement of RANTES and TARC in antitumor immune responses. Mol Ther 14:599
30. Nakazaki Y, Tani K, Lin ZT, Sumimoto H, Hibino H, Tanabe T, Wu MS, Izawa K, Hase H, Takahashi S, Tojo A, Azuma M, Hamada H, Mori S, Asano S (1998) Vaccine effect of granulocyte-macrophage colony-stimulating factor or CD80 gene-transduced murine hematopoietic tumor cells and their cooperative enhancement of antitumor immunity. Gene Ther 5:1355
31. Pardoll D (2003) Does the immune system see tumors as foreign or self? Annu Rev Immunol 21:807
32. Pasare C, Medzhitov R (2003) Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299:1033
33. Robertson MJ, Ritz J (1990) Biology and clinical relevance of human natural killer cells. Blood 76:2421
34. Romani N, Kampgen E, Koch F, Heufler C, Schuler G (1990) Dendritic cell production of cytokines and responses to cytokines. Int Rev Immunol 6:151
35. Salgia R, Lynch T, Skarin A, Lucca J, Lynch C, Jung K, Hodi FS, Jaklitsch M, Mentzer S, Swanson S, Lukanich J, Bueno R, Wain J, Mathisen D, Wright C, Fidias P, Donahue D, Clift S, Hardy S, Neuberg D, Mulligan R, Webb I, Sugarbaker D, Mihm M, Dranoff G (2003) Vaccination with irradiated autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor augments antitumor immunity in some patients with metastatic non-small-cell lung carcinoma. J Clin Oncol 21:624
36. Schaniel C, Sallusto F, Ruedl C, Sideras P, Melchers F, Rolink AG (1999) Three chemokines with potential functions in T lymphocyte-independent and -dependent B lymphocyte stimulation. Eur J Immunol 29:2934
37. Shibata S, Okano S, Yonemitsu Y, Onimaru M, Sata S, Nagata-Takeshita H, Inoue M, Zhu T, Hasegawa M, Moroi Y, Furue M, Sueishi K (2006) Induction of efficient antitumor immunity using dendritic cells activated by recombinant Sendai virus and its modulation by exogenous IFN-beta gene. J Immunol 177:3564
38. Simons JW, Jaffee EM, Weber CE, Levitsky HI, Nelson WG, Carducci MA, Lazenby AJ, Cohen LK, Finn CC, Clift SM, Hauda KM, Beck LA, Leiferman KM, Owens AH Jr, Piantadosi S, Dranoff G, Mulligan RC, Pardoll DM, Marshall FF (1997) Bioactivity of autologous irradiated renal cell carcinoma vaccines generated by ex vivo granulocyte-macrophage colony-stimulating factor gene transfer. Cancer Res 57:1537
39. Simons JW, Mikhak B, Chang JF, DeMarzo AM, Carducci MA, Lim M, Weber CE, Baccala AA, Goemann MA, Clift SM, Ando DG, Levitsky HI, Cohen LK, Sanda MG, Mulligan RC, Partin AW, Carter HB, Piantadosi S, Marshall FF, Nelson WG (1999) Induction of immunity to prostate cancer antigens: results of a clinical trial of vaccination with irradiated autologous prostate tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor using ex vivo gene transfer. Cancer Res 59:5160
40. Soiffer R, Lynch T, Mihm M, Jung K, Rhuda C, Schmollinger JC, Hodi FS, Liebster L, Lam P, Mentzer S, Singer S, Tanabe KK, Cosimi AB, Duda R, Sober A, Bhan A, Daley J, Neuberg D, Parry G, Rokovich J, Richards L, Drayer J, Berns A, Clift S, Cohen LK, Mulligan RC, Dranoff G (1998) Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. Proc Natl Acad Sci USA 95:13141
41. Steinman RM, Witmer-Pack M, Inaba K (1993) Dendritic cells: antigen presentation, accessory function and clinical relevance. Adv Exp Med Biol 329:1
42. Sumida SM, McKay PF, Truitt DM, Kishko MG, Arthur JC, Seaman MS, Jackson SS, Gorgone DA, Lifton MA, Letvin NL, Barouch DH (2004) Recruitment and expansion of dendritic cells in vivo potentiate the immunogenicity of plasmid DNA vaccines. J Clin Invest 114:1334
43. Tan JK, O'Neill HC (2005) Maturation requirements for dendritic cells in T cell stimulation leading to tolerance versus immunity. J Leukoc Biol 78:319
44. Tani K, Azuma M, Nakazaki Y, Oyaizu N, Hase H, Ohata J, Takahashi K, OiwaMonna M, Hanazawa K, Wakumoto Y, Kawai K, Noguchi M, Soda Y, Kunisaki R, Watari K, Takahashi S, Machida U, Satoh N, Tojo A, Maekawa T, Eriguchi M, Tomikawa S, Tahara H, Inoue Y, Yoshikawa H, Yamada Y, Iwamoto A, Hamada H, Yamashita N, Okumura K, Kakizoe T, Akazà H, Fujime M, Clift S, Ando D, Mulligan R, Asano S (2004) Phase I study of autologous tumor vaccines transduced with the GM-CSF gene in four patients with stage IV renal cell cancer in Japan: clinical and immunological findings. Mol Ther 10:799
45. Witmer-Pack MD, Olivier W, Valinsky J, Schuler G, Steinman RM (1987) Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. J Exp Med 166:1484

Leukemia (2008) 00, 1–8
© 2008 Nature Publishing Group All rights reserved 0887-6924/08 $30.00
www.nature.com/leu

ORIGINAL ARTICLE

RNAi-mediated silencing of p190$^{Bcr\text{-}Abl}$ inactivates Stat5 and cooperates with imatinib mesylate and 17-allylamino-17-demetoxygeldanamycin in selective killing of p190$^{Bcr\text{-}Abl}$-expressing leukemia cells

M Futami[1], T Hatano[2], Y Soda[1], S Kobayashi[1], M Miyagishi[3] and A Tojo[1]

[1]*Division of Molecular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan;* [2]*Department of Chemistry and Biotechnology, School of Engineering, University of Tokyo, Tokyo, Japan and* [3]*The 21st Century Center of Excellence (COE) Program, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*

The 190 kD (p190) and 210 kD (p210) Bcr-Abl proteins are responsible for the pathophysiology of Philadelphia chromosome (Ph)$^+$ leukemia. We applied RNA interference (RNAi) to specific killing of p190$^+$ cells, and determined the optimal sequences for gene silencing in the BCR, junctional and ABL regions of p190, respectively. Then, p190$^+$ and p210$^+$ cells were infected with lentiviral vectors encoding these shRNAs, resulting in efficient killing of p190$^+$ cells, while p210$^+$ cells were only sensitive to shBCR and shABL. In p190-transformed Ba/F3 cells, silencing of p190 specifically inhibited tyrosine phospohorylation of stat5 prior to their death, but did not affect phosphorylation of Jak2, Akt or MEK1/2. In contrast, downregulation of p190 by their treatment with 17-allylamino-17-demetoxygeldanamycin (17-AAG) was associated with reduced protein levels of Jak2, Akt and MEK1/2. shRNA targeting p190 collaborated additively with imatinib and 17-AAG in growth inhibition of Ba/F3-p190wt and imatinib-resistant Ba/F3-p190Y253H cells. Collectively, RNAi-mediated silencing of p190 is a promising option both for delineating signal transduction and for therapeutic application in 190$^+$ leukemia.
Leukemia (2008) **0,** 000–000. doi:10.1038/leu.2008.60
Keywords: Bcr-Abl; Ph$^+$ALL; RNAi; lentiviral vector; 17-AAG; Stat5

Introduction

Philadelphia chromosome (Ph)$^+$ leukemia, including chronic myeloid leukemia (CML) and acute lymphoblastic leukemia (Ph$^+$ALL), originates from hematopoietic stem/progenitor cells affected by the BCR-ABL fusion gene, which encodes constitutively active tyrosine kinase essential for development and progression of the diseases.[1] There are two major forms of Bcr-Abl protein, p210 and p190. The former is associated with almost all cases of CML and less than half of Ph$^+$ALL cases, whereas the latter mainly causes Ph$^+$ALL except for very rare cases of CML.[2]

The development of imatinib (also referred to as STI571), and the second generation tyrosine kinase inhibitors have greatly improved the clinical outcome of CML.[3–5] However, Ph$^+$ALL shows a much less durable response to these kinase inhibitors, and easily acquires resistance, caused primarily by point mutations in the Abl kinase domain such as T315I.[6,7] To overcome the resistance, the downregulation of Bcr-Abl proteins is a promising strategy for eliminating Ph$^+$ALL cells, irrespective of point mutations. From this viewpoint, we have previously reported that lentiviral delivery of anti-p190 maxizyme specifically induces apoptosis in Ph$^+$ALL cells.[8]

Recently, RNA interference (RNAi) has been recognized as a more powerful tool in the selective gene silencing. RNAi allows downregulation of target genes at the post-transcriptional level via sequence-specific mRNA depletion. This can be accomplished by the delivery of double-strand RNA in the form of small interfering RNA (siRNA) or small hairpin RNA (shRNA).[9,10] Anti-p210 RNAi targeting sequences of the junctional domain of Bcr-Abl suppressed its expression in CML cell lines, resulting in growth inhibition,[11,12] and lentivirus-mediated stable expression of anti-p210 shRNA has also been successful.[13,14] Similarly, electroporation of p190$^+$ cells with anti-p190 siRNA downregulated p190 and reduced cell viability in a dose-dependent fashion.[15] However, this procedure may exert nonspecific toxicity on intact cells, and short-lived siRNA is not suitable for monitoring cell viability. Therefore, to precisely evaluate the consequences of RNAi-mediated p190 silencing, viral vector-mediated transfer and stable expression of shRNA in p190$^+$ cells are required.

In the present study, we successfully downregulated p190 by lentiviral transduction with shRNA targeting p190, resulting in efficiently killing of p190$^+$ cells. We also observed that silencing of p190 caused specific inactivation of Stat5 and cooperated with imatinib and 17-allylamino-17-demetoxygeldanamycin (17-AAG) in the selective killing of p190$^+$ cells.

Materials and methods

Design of shRNAs targeting p190

Three types of 21-bp shRNA were designed, and named as shE1A2, shABL and shBCR, respectively. shE1A2 targets junctional lesion between BCR exon 1 and ABL exon 2 (e1a2) in p190 mRNA. The sequence was determined after the screening by a transient transfection of 293/p190 cells with a series of U6-promoter driven shE1A2 expression vectors, in which we found that the center of junctional lesion was suitable for target site (Supplementary Figure 1). We also designed shABL targeting nt 1238–1258 of ABL, and shBCR targeting nt 992-1012 of BCR, with a computer algorithm (iGENE Therapeutics, Tsukuba, Japan) to identify optimal sequences, and tested for their ability to downregulate p190 (Supplementary Figure 2). shLUC targeting *Renilla* luciferase was prepared as a control. Although shE1A2 is supposed to be specific for p190, shABL is predicted to affect not only p190 but also p210 and Abl.

Correspondence: Dr A Tojo, Division of Molecular Therapy, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Munato-ku, Tokyo 108-8639, Japan.
E-mail: a-tojo@ims.u-tokyo.ac.jp
Received 26 August 2007; revised 18 February 2008; accepted 19 February 2008

Gml : Template: Ver 1.1.3 | Journal: LEU | Article : npg_leu_leu200860 | ☐ Disk used | Pages: 1–8 | Despatch Date: 3/3/2008 | Op: XXX Ed: xxx CE: xxx Graphic: xxx

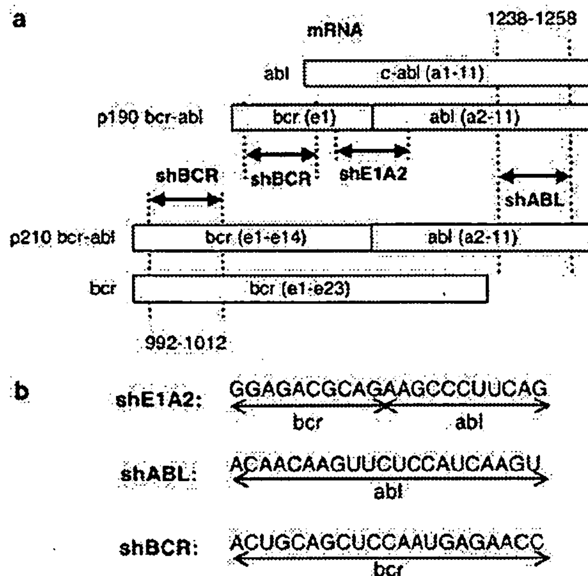

Figure 1 Target sites for shRNAs. (a) Locations of target site. Arrows indicate the target sites of shRNAs on abl, e1a2 of p190 Bcr, e14a2 of p210 Bcr-Abl and bcr mRNA. (b) The target site sequences. Arrows indicate areas in which the sequence is complementary to that of bcr or abl.

Similarly, shBCR is predicted to affect p190, p210 as well as Bcr (Figures 1a and b).

Production of lentiviral vectors

The nucleotide sequences of the shRNA expression cassette in the piGENE-PURhU6/shRNA were amplified by polymerase chain reaction using Platinum *pfx* DNA polymerase (Invitrogen) and a primer set containing a *Bam*HI site (forward 5′-ATGGATCCAAGGTCGGGCAGGAAGAGG-3′ and reverse 5′-ATGGATCCCATGATGATTACGCCAAGCTTGCAT-3′). The resulting fragments were digested with *Bam*HI and ligated into a *Bam*HI-digested self-inactivating lentiviral transfer vector. The expression cassettes for shE1A2, shABL and shLUC were inserted into lentiviral transfer vector pCS-CDF-EG-PRE containing elongation factor 1α (EF1α) promoter-driven human CD2 as a selection marker (pHIV-CD2), and the shBCR expression cassette was inserted into a similar transfer vector containing CD271 as a marker (pHIV-CD271). Lentiviral vector particles were produced by cotransfection of 293T cells with pHIV-hU6/shRNA, pMDLg/p.RRE, pRSV-rev and pMD.G as described previously.[16] Transduction efficiency was analyzed by flow cytometry of marker gene expression, resulting in 94–99% at high multiplicity of infection (MOI = 5) and around 50% (36–59%) at low MOI (MOI = 1).

Western blot analysis

Cells were lysed, and 50 μg of the lysate were electrophoresed in SDS-polyacrylamide gels, transferred onto polyvinylidene fluoride membranes, and blotted with the respective antibodies, according to the manufactures' instructions. All secondary antibodies were peroxidase-conjugated, and proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA). The following antibodies were used: anti-Abl (8E9; BD Biosciences), anti-Bcr (N-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Jak2 (06-255; Upstate Biotechnology, Lake Placid, NY, USA), anti-phospho-Jak2 (Tyr1007/1008; Upstate Biotechnology), and anti-Stat5 (C-17; Santa Cruz Biotechnology); anti-phospho-Stat5 (Tyr694), anti-Akt1, (2H10) anti-phospho-Akt (Ser473), anti-MEK1/2 (L38C12), anti-phospho-MEK1/2 (Ser221) (all from Cell Signaling Technology, Danvers, MA, USA); anti-Actin (AC-40; Sigma, St Louis, MO, USA), sheep anti-mouse IgG (515-036-072; Jacson ImmunoResearch Labolatories, West Grove, PA, USA) and donkey anti-rabbit IgG (NA934V; Amersham Biosciences, Little Chalfont, Bucks, UK).

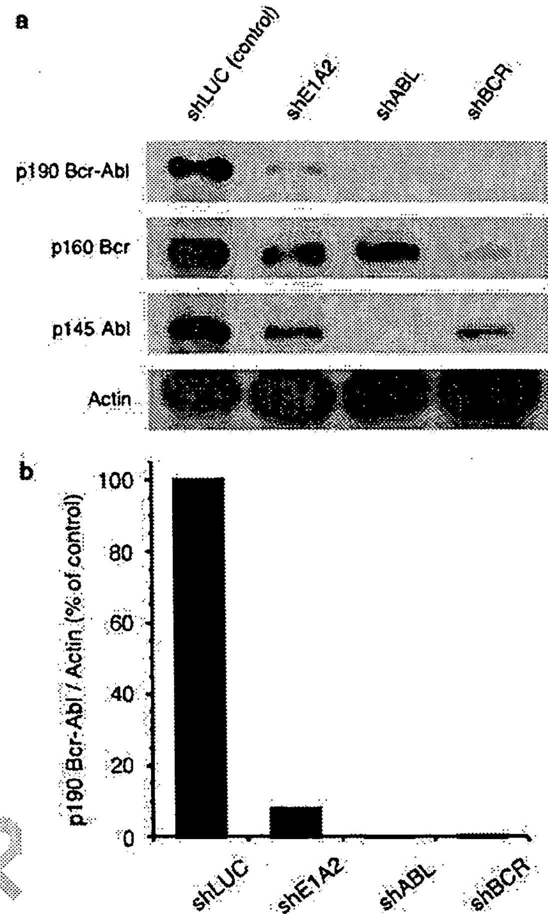

Figure 2 Lentivirus-mediated RNAi silences the expression of p190 Bcr-Abl. (a) HEK 293/p190 cells were transduced with shLUC (control), shE1A2, shABL and shBCR at an MOI of 5. The cells were lysed on day 3, and lysates were labeled with the respective antibodies: p190 Bcr-Abl and p145 Abl, with anti-Abl antibody; p160 Bcr, with anti-Bcr antibody and and actin, with anti-actin antibody. The transduction efficiencies, determined by FACS analysis of marker gene expression on day 3: shLUC, 99.1%; shE1A2, 99.3%; shABL, 96.9% and shBCR, 98.1%. (b) Quantitative analysis of p190 expression. Quantitative analysis was performed using NIH Image 1.63 (National Institutes of Health, Bethesda, MD, USA), and p190 levels were normalized by actin.

Cell count and viability assay

Aliquots of 5×10^4 cells (10^5 cells for KOPN-30 cells) in 1 ml of culture medium were plated in a 24-multiwell plate. Cell number and viability were determined by trypan blue dye

exclusion. For cell proliferation assay, 10^3 cells in 100 μl of culture medium were grown in a 96-multiwell plate. Viable cell number was assessed on day 4 using WST-8 assay kit (Seikagaku, Tokyo, Japan).

Colony-forming assay
Human CD34$^+$ cells were purified from umbilical cord blood mononuclear cells by a two-round separation procedure using the Indirect CD34 MicroBead Kit (Miltenyi Biotec). Purified CD34$^+$ cells were cultured in α-minimal essential medium containing 30% fetal bovine serum (FBS), 50 ng ml^{-1} hSCF, 50 ng ml^{-1} hTPO, 10 ng ml^{-1} hIL-3 and 50 ng ml^{-1} Flt3L for 24 h, transduced with anti-p190 shRNA lentiviral vectors at a MOI of 20, and incubated for 48 h. Aliquots of 10^3 transduced cells were plated in triplicate in 1 ml of 0.9% methylcellulose containing 30% FBS, 50 ng ml^{-1} hSCF, 10 ng ml^{-1} hIL-3, 100 ng ml^{-1} hG-CSF, and 2 U ml^{-1} hEPO. After 14 days, the number of each type of colony was scored.

Q2

Results

Silencing effect of anti-p190 shRNA lentiviral vectors
To confirm the silencing effect, we transduced HEK 293/p190 cells with lentiviral vectors encoding anti-p190 shRNA (shE1A2, shABL and shBCR) at a MOI of 5, and the expression level of p190 was determined by western blot analysis. p190 Bcr-Abl expression were substantially suppressed (0.0–8.0%) by these three anti-p190 shRNAs (Figures 2a and b). The expression of p145 Abl and p160 Bcr was substantially inhibited by shABL and shBCR, respectively (Figure 2a).

Proliferation and survival of leukemia cell lines after the transduction of shRNAs
To investigate the biological consequences of anti-p190 shRNAs, p190$^+$ KOPN-30 cells, p210$^+$ K562 cells and p190/p210$^-$ NALM-6 cells were infected with lentiviral vectors encoding these shRNAs at an MOI of 5, and cell proliferation and viability were determined by trypan blue dye exclusion. The Ph$^+$ALL-derived KOPN-30 p190$^+$ cells ceased proliferation at two days after transduction with shE1A2, shABL and shBCR, and decreased in cell number and viability in a time-dependent manner, confirming that shRNA-mediated downregulation of p190 can kill Ph$^+$ALL cells (Figure 3). Given that shABL and shBCR would be expected to knock down both p190 and p210, we assessed the effect of these shRNAs in K562 cells, which express p210. As expected, shABL and shBCR eradicated the K562 cells, whereas shE1A2 did not have a notable effect, indicating that shE1A2 is sequence-specific to p190 (Figure 3). To further confirm that RNAi-mediated downregulation is target sequence specific, we also transduced p190/p210$^-$ NALM-6 cells. None of the anti-p190 shRNAs affected survival in NALM-6 cells, although shABL appeared to have some inhibitory effect on growth, which suggests that p145 Abl may be positively involved in NALM-6 cell proliferation.

The effect of shRNAs on imatinib-resistant cells
To determine whether anti-p190 shRNAs overcome imatinib resistance, murine Ba/F3 cells transformed with wild-type p190 or imatinib-resistant mutant (p190Y253H) were transduced with shE1A2, shABL and shBCR. In the absence of IL-3, these shRNAs depleted BaF/3 cells with both types of p190 in an equipotent manner (Figure 4 left). Supplementation of IL-3 clearly rescued these cells from shRNA-mediated cytotoxic effects, suggesting that signal transduction machinery downstream of IL-3 receptor might be maintained irrespective of p190 expression (Figure 4 right).

The effect of downregulation of Abl and Bcr on normal hematopoiesis
We tested the effects of shABL and shBCR on cytokine-dependent clonal growth and differentiation of normal CD34$^+$ cells. Figure 4b shows that shBCR did not influence colony formation derived from colony-forming unit granulocyte-ery-

UNCORRECTED PROOF

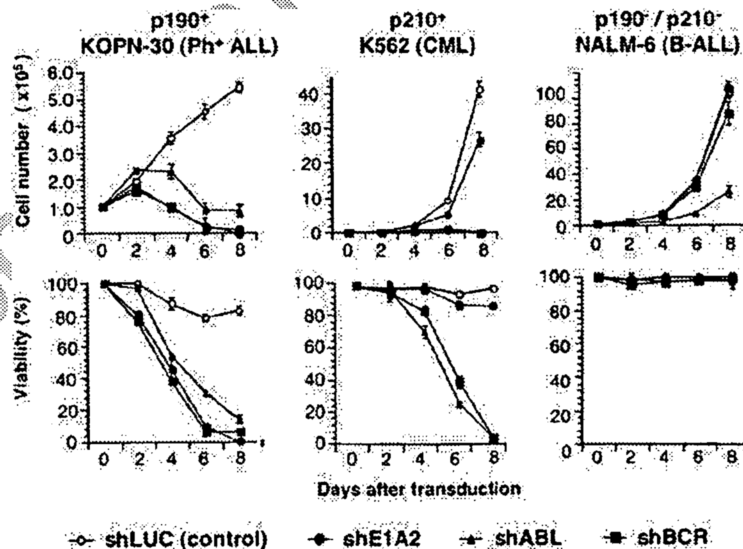

Figure 3 RNAi targeting the junctional domain of p190 kills specifically p190$^+$ cells; RNAi targeting Abl or Bcr kills both p190$^+$ cells and p210$^+$ cells. Cells were transduced with shLUC (control), shE1A2, shABL and shBCR at an MOI of 5. Cell number and viability was determined by the trypan blue exclusion.

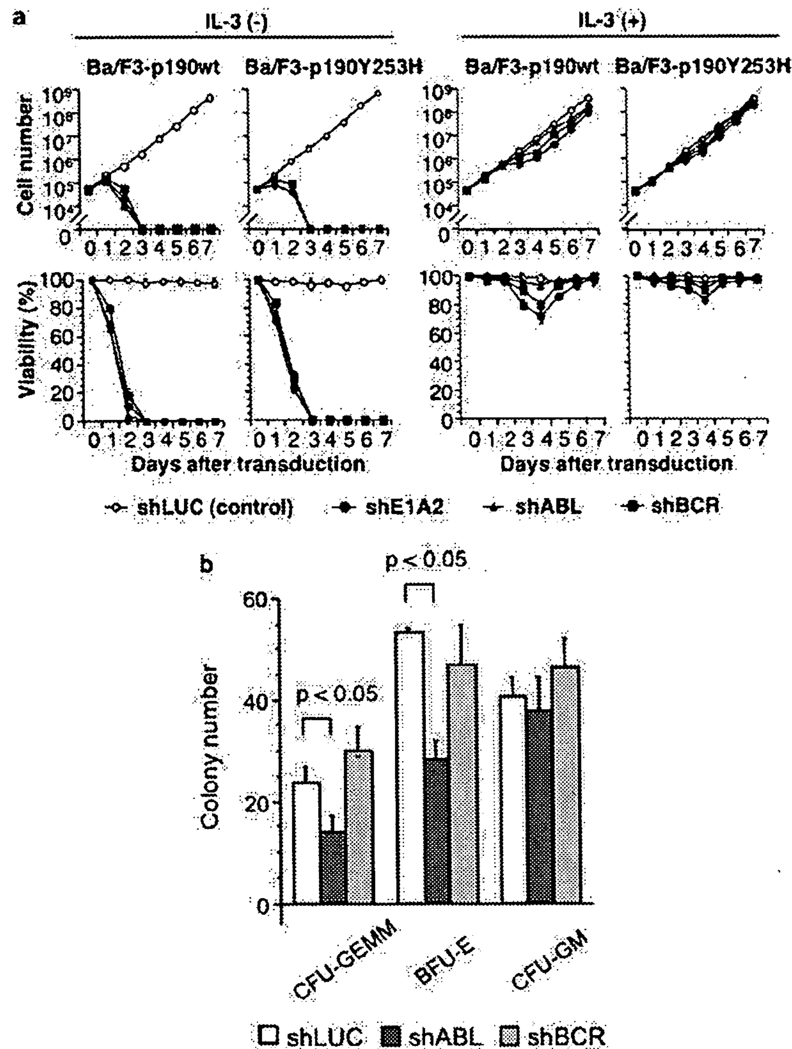

Figure 4 (a) Anti-p190 shRNAs kill BaF/3-p190 cells, and supplementation with IL-3 rescues these cells. Cells were transduced with shLUC, shE1A2, shABL and shBCR at an MOI of 5, and cultured in the absence or presence (10 ng ml^{-1}) of murine IL-3. Cell number and viability were determined by trypan blue exclusion. (b) Knock-down of Abl suppresses hematopoietic colony formation of normal cord blood CD34$^+$ cells. Cord blood CD34$^+$ cells were transduced with shRNA lentiviral vectors at an MOI of 20, and cytokine-dependent clonal growth was examined by the methylcellulose hematopoietic colony-forming assay. The number of various types of colonies was scored after 14 days in culture.

throcyte-monocyte-megakariocyte (CFU-GEMM), CFU granulocyte-monocyte (CFU-GM), and burst-forming unit erythroid (BFU-E) cells, but shABL markedly suppressed CFU-GEMM and BFU-E-derived colony formation.

The effect of Anti-p190 shRNA on p190 and the downstream signal transduction pathway

Bcr-Abl kinase activates a number of signal transduction pathways, including the Jak/Stat, PI3K/Akt and Ras/Raf/MEK/ERK pathways.[17] However, the relative contributions of these signaling pathways to p190-triggered proliferation and survival are not fully understood. To elucidate the primary event that is directly influenced by the downregulation of p190 Bcr-Abl, Ba/F3-p190wt cells were transduced with anti-p190 shRNAs, and the phosphorylation status of molecules involved in Bcr-Abl-mediated transformation was determined by immunoblot analysis at 48 h after transduction (Figure 5a). There were no significant changes in phosphorylation status of Jak2 by the treatment with anti-p190 shRNAs. In contrast, a marked reduction in phosphorylated Stat5 was noted in anti-p190 shRNA-treated BaF/3-p190wt cells, supporting the idea that p190 activates Stat5 in a Jak2-independent manner. Anti-p190 shRNAs did not significantly affect phosphorylation of Akt or MEK1/2. These results imply that downregulation of p190 rapidly results in the inactivation of Stat5, but inactivation of either PI3K/Akt or Ras/Raf/MEK/ERK pathway is not significant compared with Stat5.

The effect of 17-AAG on p190 and the downstream signal transduction pathway

Treatment with 17-AAG disrupts Hsp90 function and degrades its client protein, p190/p210.[18,19] Ba/F3-p190wt cells were treated with increasing concentrations of 17-AAG for 24 h, and the expression level and phosphorylation status of the same

NPG.LEU.LEU200860

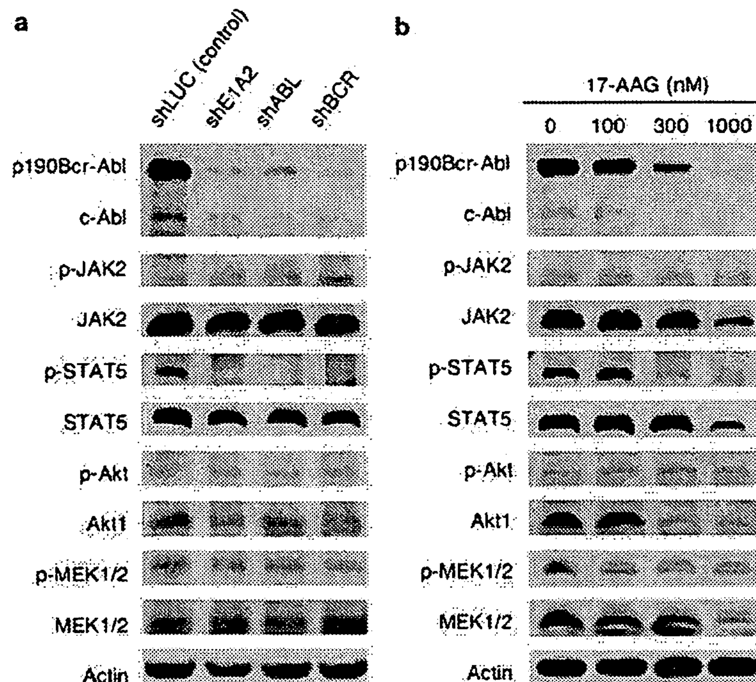

Figure 5 Western blot analysis of Ba/F3-p190wt cells. (a) Ba/F3-p190wt cells were transduced with shRNA lentiviral vectors at an MOI of 5. Cells were harvested at 48 h after transduction, lysed and labeled with the respective antibodies. (b) Ba/F3-p190wt cells were cultured in the presence or absence of 17-AAG (0–1000 nM) for 24 h. Lysates were labeled with the respective antibodies.

molecules shown in Figure 5a were analyzed. As shown in Figure 5b, 17-AAG downregulated both p190 and p145 Abl in a dose-dependent manner at concentrations >300 nM. The Jak2 protein level was downregulated at 1000 nM. The downregulation of phosphorylated Stat5 preceded that of total Stat5 protein level, which was not affected by the treatment with 17-AAG up to 300 nM. Akt1 and MEK1/2 were downregulated by 17-AAG at around 300 nM. These results suggest that 17-AAG affects p190 as well as various molecules involved in Bcr-Abl-mediated transformation.

Combined effects between shRNA, imatinib and 17-AAG

Because anti-p190 RNAi and 17-AAG can downregulate p190 by different mechanisms, and imatinib inactivates the kinase activity of p190, their combination may work in a synergistic or additive manner in killing of Ba/F3-p190wt and BaF/3-p190Y253H cells. These two cell lines were transduced with anti-p190 shRNAs in a suboptimal condition (MOI = 1) and cultured with increasing dose of either imatinib or 17-AAG. Under these conditions, anti-p190 shRNAs increased sensitivity of Ba/F3-p190wt cells to imatinib and 17-AAG. BaF/3-p190Y253H cells did not respond to imatinib, even at 5 μM; however, the anti-p190 shRNAs appeared to restore sensitivity to higher concentrations of imatinib (Figure 6a). On the other hand, anti-p190 shRNAs cooperated with 17-AAG to kill Ba/F3-p190Y253H cells, with a dose-response curve similar to that in Ba/F3-p190wt cells (Figure 6b). When Ba/F3-p190 cells were exposed to various dosage combinations of 17-AAG and imatinib, the drugs acted synergistically to kill Ba/F3-p190wt cells, and 17-AAG clearly sensitized Ba/F3-p190Y253H cells to imatinib (Figure 6c).

Discussion

We applied RNAi to test whether specific and efficient killing of Ph^+ALL cells could be achieved by downregulation of p190. Wohlbold *et al.*[15] first reported the effective inhibition of p190 protein by siRNA and its cytotoxicity in murine 32D-p190 cells; however, they induced siRNA by electroporation, which is likely to cause nonspecific damage to recipient cells. We independently designed a series of overlapping shRNAs complementary to the e1a2 junction of p190 and determined the optimal target sequence for inhibition, finding a sequence identical to that described by Wohlbold *et al.* Use of lentiviral transfer of shRNA expression cassettes provided efficient transduction and sustained silencing of the target protein with reduced nonspecific damage. Using a similar vector system, Scherr *et al.*[14] have demonstrated that stable, but not transient, RNAi can efficiently deplete p210 from $p210^+$ cells, depending on the MOI used for infection; transduction at a high MOI resulted in almost complete loss of viable cells, but transduction at a low MOI caused minimal growth inhibition. These results are compatible with our data in Figures 4 and 6.

We also showed that shBCR and shABL very efficiently downregulated p190/p210 Bcr-Abl proteins as well as their normal counterparts. Although Abl-deficient mice have multiple defects, including high postnatal mortality, runting and morphological abnormalities, Abl is considered to be dispensable for the function of hematopoietic stem cells, myeloid progenitor cells and immune cells.[20,21] On the other hand, Bcr-null mice appear intact, and a peculiar finding is that their neutrophils show a marked increase in superoxide production upon activation.[22] Consistent with these findings, shABL slightly inhibited proliferation of $p190/p210^-$ NALM-6 cells without loss of viability, but shBCR did not affect these cells. In addition, BaF/3-p190 cells treated with shABL and shBCR could be

UNCORRECTED PROOF

NPG.LEU.LEU200860

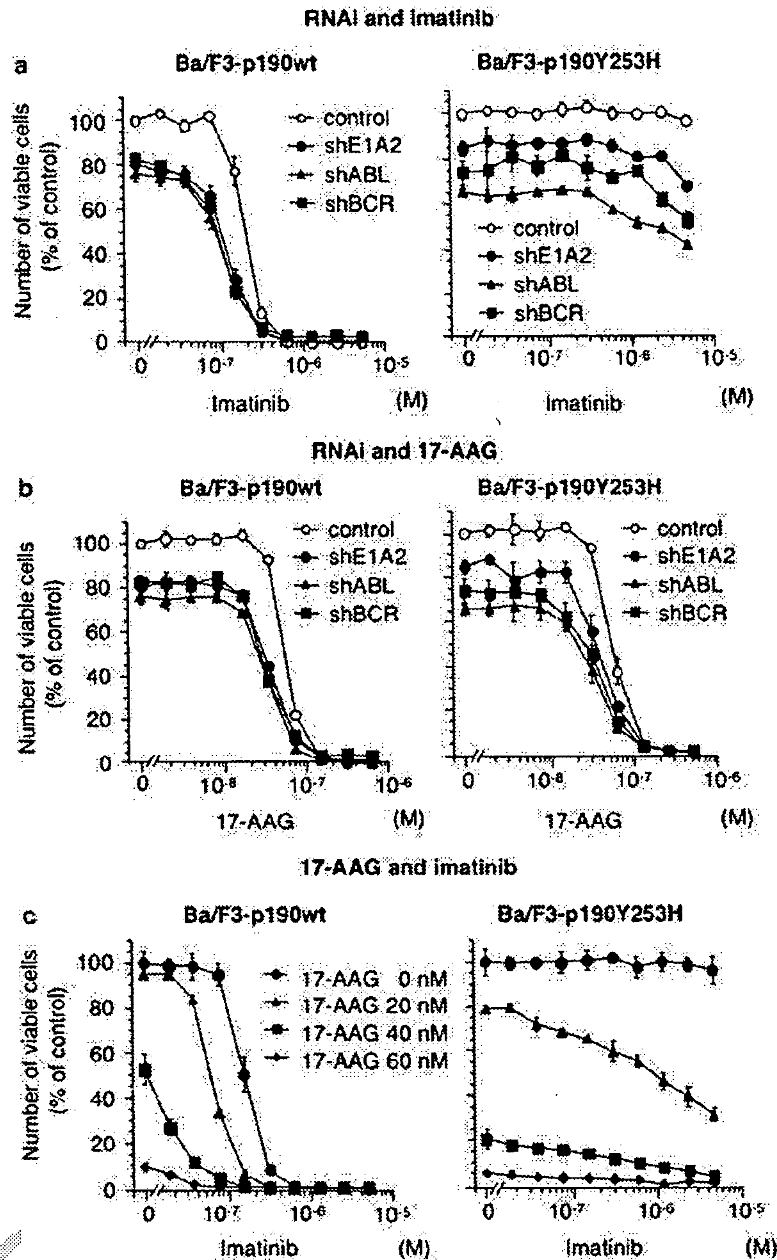

Figure 6 Cell proliferation assay conducted after combined treatment with shRNAs and imatinib or 17-AAG. (**a**) Combination of shRNAs and imatinib. Ba/F3-p190wt and Ba/F3-p190Y253H cells were transduced with shRNA lentiviral vectors under suboptimal condition (MOI = 1) and cultured in the presence or absence of imatinib (0–5 μM). (**b**) Combination of shRNAs and 17-AAG. Ba/F3-p190wt cells and Ba/F3-p190Y253H cells were transduced with shRNA lentiviral vectors at an MOI of 1 and cultured in the presence or absence of 17-AAG (0–500 nM). (**c**) Combination of 17-AAG and imatinib. Ba/F3-p190wt cells and Ba/F3-p190Y253H cells were treated with various combinations of 17-AAG (0–60 nM) and imatinib (0–5 μM). Viable cell number was determined using a WST-8 assay after the 96 h of treatment.

rescued by exogenous IL-3, indicating that neither Abl nor Bcr is essential for response to IL-3. In addition, shABL, but not shBCR, inhibited CFU-GEMM/BFU-E-derived colony formation, which suggests that Abl plays a role especially in normal erythropoiesis. Furthermore, shABL, but not shBCR, profoundly depleted 293 cells during the culture of several days, suggesting the tissue-specific critical role of Abl (data not shown). Thus, it is possible that, with an improved delivery system such as our previously developed CD19-targeted liposomes,[23] anti-p190 shRNAs may become a therapeutic option in Ph$^+$ ALL.

Hsp90 is a molecular chaperon that forms complexes with a variety of polypeptides and facilitates the initial folding and stabilization of its client proteins. Hsp90 is disrupted by 17-AAG,[24] which induces the degradation of p190/p210[18,19] and thereby inhibits the proliferation of p190/p210$^+$ cells with or without the kinase domain mutation.[25] We revealed that 17-AAG downregulated p190 and other signaling molecules downstream of p190. Some studies have shown that anti-p210 siRNA in combination with imatinib or 17-AAG exerts more potent activity than control siRNA with imatinib or 17-AAG in

p210$^+$ cells, with or without the kinase domain mutation.[12,26] In addition, Radujkovic *et al.*[27] have shown that combination treatment with 17-AAG and imatinib inhibits cell proliferation additively/antagonistically in imatinib-sensitive p210$^+$ cells and synergistically in imatinib-resistant cells. Our results are closely compatible with these data, and combination-targeting strategies, as described here, may have enhanced therapeutic potency.

The activation of Stat5 in Ph$^+$ leukemia cells is well recognized,[28,29] but its significance in the pathogenesis of Ph$^+$ leukemia is controversial. However, BCR-ABL-transduced hematopoietic progenitors from Stat5-null mice cannot generate leukemia in recipient mice,[30] and that anti-Stat5 siRNA impairs Ph$^+$ myeloid colony formation in CML.[31] These two recent studies suggest that Stat5 contributes to Bcr-Abl-induced leukemogenesis. Bcr-Abl may directly activate Stat5 through phosphorylation,[32,33] or may indirectly activate Stat5 through phosphorylation by Jak2 or Src family kinases,[28,34,35] both of which are activated in Bcr-Abl-expressing cells. Interestingly, tyrosine phosphorylation of Stat5 was substantially eliminated in BaF/3-p190wt cells transduced with anti-p190 shRNAs, whereas Jak2, Akt and MEK1/2 were still in an activated state. These results offer further evidence for the critical role of Stat5 in Bcr-Abl-induced transformation of hematopoietic cells. It is likely that p190 directly phosphorylates Stat5 and that Jak2 is remote from activation of Stat5 in this cell context, although the possible involvement of Src family kinases such as Hck and Lyn cannot be excluded. The mechanism by which phophorylation of several signal transducers was maintained after p190 downregulation remains to be elucidated. Nevertheless, compared with the PI3K/Akt and Ras/Raf/MEK/ERK pathways, the Stat5 signaling pathway contributes more closely to p190-mediated transformation of hematopoietic cells.

In conclusion, RNAi-mediated silencing of p190 is a promising tool for both signal transduction delineation and therapeutic application in p190-expressing leukemia.

Acknowledgements

We thank Dr IM Verma (Salk Institute, La Jolla, CA, USA) and Cell Genesys for providing HIV vector constructs. We are indebted to Mr K Takahashi, Ms S Suzuki and Ms M Oiwa for technical support.

UNCORRECTED

References

1 Lugo TG, Pendergast AM, Muller AJ, Witte ON. Tyrosine kinase activity and transformation potency of bcr-abl oncogene products. *Science* 1990; **247**: 1079–1082.

2 Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 1996; **88**: 2375–2384.

3 Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S *et al.* Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 1996; **2**: 561–566.

4 Weisberg E, Manley PW, Breitenstein W, Bruggen J, Cowan-Jacob SW, Ray A *et al.* Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. *Cancer Cell* 2005; 7: 129–141.

5 Lombardo LJ, Lee FY, Chen P, Norris D, Barrish JC, Behnia K *et al.* Discovery of N-(2-chloro-6-methyl- phenyl)-2-(6-(4-(2-hydroxyethyl)- piperazin-1-yl)-2-methylpyrimidin-4- ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem* 2004; **47**: 6658–6661.

6 von Bubnoff N, Peschel C, Duyster J. Resistance of Philadelphia-chromosome positive leukemia towards the kinase inhibitor imatinib (STI571, Glivec): a targeted oncoprotein strikes back. *Leukemia* 2003; **17**: 829–838.

7 Deininger M, Buchdunger E, Druker BJ. The development of imatinib as a therapeutic agent for chronic myeloid leukemia. *Blood* 2005; **105**: 2640–2653.

8 Soda Y, Tani K, Bai Y, Saiki M, Chen M, Izawa K *et al.* A novel maxizyme vector targeting a bcr-abl fusion gene induced specific cell death in Philadelphia chromosome-positive acute lymphoblastic leukemia. *Blood* 2004; **104**: 356–363.

9 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494–498.

10 Hannon GJ. RNA interference. *Nature* 2002; **418**: 244–251.

11 Wilda M, Fuchs U, Wossmann W, Borkhardt A. Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* 2002; **21**: 5716–5724.

12 Wohlbold L, van der Kuip H, Miething C, Vornlocher HP, Knabbe C, Duyster J *et al.* Inhibition of bcr-abl gene expression by small interfering RNA sensitizes for imatinib mesylate (STI571). *Blood* 2003; **102**: 2236–2239.

13 Li MJ, McMahon R, Snyder DS, Yee JK, Rossi JJ. Specific killing of Ph+ chronic myeloid leukemia cells by a lentiviral vector-delivered anti-bcr/abl small hairpin RNA. *Oligonucleotides* 2003; **13**: 401–409.

14 Scherr M, Battmer K, Schultheis B, Ganser A, Eder M. Stable RNA interference (RNAi) as an option for anti-bcr-abl therapy. *Gene Therapy* 2005; **12**: 12–21.

15 Wohlbold L, van der Kuip H, Moehring A, Granot G, Oren M, Vornlocher HP *et al.* All common p210 and p190 Bcr-abl variants can be targeted by RNA interference. *Leukemia* 2005; **19**: 290–292.

16 Bai Y, Soda Y, Izawa K, Tanabe T, Kang X, Tojo A *et al.* Effective transduction and stable transgene expression in human blood cells by a third-generation lentiviral vector. *Gene Therapy* 2003; **10**: 1446–1457.

17 Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, McCubrey JA. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia* 2004; **18**: 189–218.

18 Nimmanapalli R, O'Bryan E, Bhalla K. Geldanamycin and its analogue 17-allylamino-17-demethoxygeldanamycin lowers Bcr-Abl levels and induces apoptosis and differentiation of Bcr-Abl-positive human leukemic blasts. *Cancer Res* 2001; **61**: 1799–1804.

19 Nimmanapalli R, O'Bryan E, Huang M, Bali P, Burnette PK, Loughran T *et al.* Molecular characterization and sensitivity of STI-571 (imatinib mesylate, Gleevec)-resistant, Bcr-Abl-positive, human acute leukemia cells to SRC kinase inhibitor PD180970 and 17-allylamino-17-demethoxygeldanamycin. *Cancer Res* 2002; **62**: 5761–5769.

20 Tybulewicz VL, Crawford CE, Jackson PK, Bronson RT, Mulligan RC. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* 1991; **65**: 1153–1163.

21 Schwartzberg PL, Stall AM, Hardin JD, Bowdish KS, Humaran T, Boast S *et al.* Mice homozygous for the ablm1 mutation show poor viability and depletion of selected B and T cell populations. *Cell* 1991; **65**: 1165–1175.

22 Voncken JW, van Schaick H, Kaartinen V, Deemer K, Coates T, Landing B *et al.* Increased neutrophil respiratory burst in bcr-null mutants. *Cell* 1995; **80**: 719–728.

23 Harata M, Soda Y, Tani K, Ooi J, Takizawa T, Chen M *et al.* CD19-targeting liposomes containing imatinib efficiently kill Philadelphia chromosome-positive acute lymphoblastic leukemia cells. *Blood* 2004; **104**: 1442–1449.

24 Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH. Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. *Cell* 1997; **90**: 65–75.

25 Gorre ME, Ellwood-Yen K, Chiosis G, Rosen N, Sawyers CL. BCR-ABL point mutants isolated from patients with imatinib mesylate-resistant chronic myeloid leukemia remain sensitive to inhibitors of the BCR-ABL chaperone heat shock protein 90. *Blood* 2002; **100**: 3041–3044.

26 Withey JM, Harvey AJ, Crompton MR. RNA interference targeting of Bcr-Abl increases chronic myeloid leukemia cell killing by 17-allylamino-17-demethoxygeldanamycin. *Leuk Res* 2006; **30**: 553–560.

27 Radujkovic A, Schad M, Topaly J, Veldwijk MR, Laufs S, Schultheis BS *et al.* Synergistic activity of imatinib and 17-AAG in imatinib-resistant CML cells overexpressing BCR-ABL–Inhibition of P-glycoprotein function by 17-AAG. *Leukemia* 2005; **19**: 1198–1206.
28 Shuai K, Halpern J, ten Hoeve J, Rao X, Sawyers CL. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. *Oncogene* 1996; **13**: 247–254.
29 Carlesso N, Frank DA, Griffin JD. Tyrosyl phosphorylation and DNA binding activity of signal transducers and activators of transcription (STAT) proteins in hematopoietic cell lines transformed by Bcr/Abl. *J Exp Med* 1996; **183**: 811–820.
30 Hoelbl A, Kovacic B, Kerenyi MA, Simma O, Warsch W, Cui Y *et al.* Clarifying the role of Stat5 in lymphoid development and Abelson-induced transformation. *Blood* 2006; **107**: 4898–4906.
31 Scherr M, Chaturvedi A, Battmer K, Dallmann I, Schultheis B, Ganser A *et al.* Enhanced sensitivity to inhibition of SHP2, STAT5, and Gab2 expression in chronic myeloid leukemia (CML). *Blood* 2006; **107**: 3279–3287.
32 Nieborowska-Skorska M, Wasik MA, Slupianek A, Salomoni P, Kitamura T, Calabretta B *et al.* Signal transducer and activator of transcription (STAT)5 activation by BCR/ABL is dependent on intact Src homology (SH)3 and SH2 domains of BCR/ABL and is required for leukemogenesis. *J Exp Med* 1999; **189**: 1229–1242.
33 Sillaber C, Gesbert F, Frank DA, Sattler M, Griffin sJD. STAT5 activation contributes to growth and viability in Bcr/Abl-transformed cells. *Blood* 2000; **95**: 2118–2125.
34 Wilson-Rawls J, Xie S, Liu J, Laneuville P, Arlinghaus RB. P210 Bcr-Abl interacts with the interleukin 3 receptor beta(c) subunit and constitutively induces its tyrosine phosphorylation. *Cancer Res* 1996; **56**: 3426–3430.
35 Klejman A, Schreiner SJ, Nieborowska-Skorska M, Slupianek A, Wilson M, Smithgall TE *et al.* The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO J* 2002; **21**: 5766–5774.

Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)

UNCORRECTED PROOF