Retroviral Transfer and Expression of the Human Multiple Drug Resistance (*MDR*) Gene in Peripheral Blood Progenitor Cells[1]

Maureen Ward, Patricia Pioli, Janet Ayello, Robert Reiss, Giuseppina Urzi,[2] Christine Richardson, Charles Hesdorffer, and Arthur Bank[3]

Columbia University, College of Physicians and Surgeons, Department of Genetics and Development and Department of Medicine, New York, New York 10032

ABSTRACT

The multiple drug resistance (*MDR*) gene P-glycoprotein product is a transmembrane efflux pump that prevents toxicity of a variety of chemotherapeutic agents, including the anthracyclines, *Vinca* alkaloids, podophyllins, and taxol. The bone marrow toxicity of these drugs is due to the low or absent expression of *MDR* in marrow cells. Transfer and expression of the human *MDR* gene into bone marrow progenitors should prevent this toxicity. We report here the efficient transfer and expression of the *MDR* gene by retroviral-mediated gene transfer into $CD34^+$ cells isolated from peripheral blood progenitor cells (PBPCs), comparable to that obtained using bone marrow-derived progenitors. Optimal *MDR* transduction of these PBPC-derived cells requires exposure to growth factors and a period of preincubation. In addition, we demonstrate that we can transduce up to 100% of progenitor cells derived from PBPCs and can protect up to 25% of these progenitors from a dose of taxol toxic to untransduced controls.

INTRODUCTION

Autologous transplantation with either BM[4]-derived cells or PBPCs is commonly used in association with intensive chemotherapy in patients with solid tumors and hematological cancers (1).

The ease of collection, possibility of multiple harvests, and increased cell yields with mobilization after administration of growth factors and/or chemotherapeutic agents (2, 3) make PBPCs a more desirable source than BM cells for hematopoietic progenitors for autologous BM transplantation. PBPC harvests and reinfusion and growth factors are used to avert severe marrow toxicity due to chemotherapy. However, the efficacy of these measures diminishes with cancer treatment, due to eventual stem cell and progenitor depletion (4). Transfer of the human *MDR1* gene into hematopoietic progenitors could provide an additive therapy to protect marrow stem cells from the myelosupressive effects of chemotherapy with agents such as the anthracyclines, *Vinca* alkaloids, podophyllins, and taxol, which depend on *MDR* activity for their efflux from cells. *MDR* encodes a transmembrane P-glycoprotein that functions as an efflux pump for these anticancer agents (5). Expression of *MDR* is low in BM cells, although there is somewhat more P-glycoprotein expression in earlier hematopoietic progenitors (6). Retroviral gene transfer of a *MDR* cDNA into mouse BM progenitors produces not only increased amounts of *MDR* RNA and protein but also the ability to enrich for *MDR*-expressing BM cells after exposure to taxol (7, 8).

We have previously reported the use of an *MDR*-containing retroviral producer line for the high-level transfer and expression of the *MDR* gene into $CD34^+$ cells derived from human BM (9). The present study uses the same safe and efficient MDR retrovirus to document a comparable level of transfer and expression of the *MDR* gene into PBPCs. In these studies, we use resistance of hematopoeitic progenitor colonies to taxol to assess the functional expression of *MDR* P-glycoprotein. The results indicate that: (*a*) optimal *MDR* transduction of $CD34^+$ PBPCs requires the presence of growth factors and preincubation; and (*b*) we can protect up to 25% of these hematopoietic progenitors from the toxic effects of taxol.

MATERIALS AND METHODS

Cell-free Supernatant Preparation. The amphotropic MDR viral producer line (A12M1) used in these studies has been described previously (8). A12M1 cells were grown in 175-cm^2 flasks (Nunc, Inc., Naperville, IL) with DMEM (Life Technologies, Inc., Gaithersburg, MD), 10% newborn calf serum, and 1% antibiotic/antimycotic (Sigma, Chemical Co., St. Louis, MO). At 80% confluence, the DMEM was removed, and the cells were washed with 1× PBS and replaced with IMDM (Life Technologies), 20% FCS, and 1% penicillin/streptomycin (Sigma). After 24 h, the viral supernatant was collected and filtered through a 0.45-μm filter and frozen at −70°C.

Isolation and FACS Analysis of $CD34^+$ Cells. Mobilized PBPCs were collected by standard apheresis techniques 8–12 days after exposure of patients to chemotherapy and granulocyte colony-stimulating factor. $CD34^+$ cells were isolated by clinical-scale CEPRATE columns using anti-CD34 antibody (CellPro, Bothell, WA; Ref. 10). $CD34^+$ cells were quantitated by FACS analysis. Five × 10^5 sorted cells were incubated for 20 min on ice with 20 μl phycoerythrin-conju

Received 10/19/95; revised 12/20/95; accepted 2/7/96.
[1] This work was supported by USPHS Grants NIH-5-R01-HL-48345, NIH-5-R37-DK-25274, NIH-5-P60-HL-28381, and NIH-1-U01-CA-65838.
[2] Supported by the Associazione Italiana per la Ricerca sul Cancro (Palermo, Italy).
[3] To whom requests for reprints should be addressed, at Columbia University, HHSC 16-1604, 701 West 168th Street, New York, NY 10032. Phone: (212) 305-4186; Fax: (212) 923-2090.
[4] The abbreviations used are: BM, bone marrow; MDR, multiple drug resistance; PBPC, peripheral blood progenitor cell; PBSC, peripheral blood stem cell; A12M1, amphotropic MDR viral producer line; IMDM, Iscove's modified Dulbecco's medium; IL, interleukin; FACS, fluorescence-activated cell sorting; BFU-E, bursting forming unit-erythroid; CFU-GM, colony forming unit-granulocyte-macrophage.

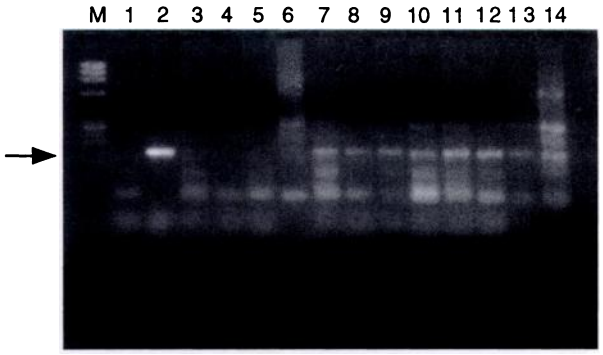

Fig. 1 *MDR* PCR analysis of BFU-Es from PBSC CD34$^+$ cells. Individual BFU-E colonies were picked from methylcellulose assays and analyzed by PCR for the transferred *MDR* cDNA. *Lane M*, PhiX marker; *Lane 1*, negative control; *Lane 2*, positive control; *Lanes 3–5*, BFU-E colonies from untransduced CD34$^+$ cells; *Lanes 6–14*, BFU-E colonies from transduced CD34$^+$ cells. *Arrow*, expected 167-bp band.

gated, anti-CD34 antibody (HPCA-2; Becton Dickinson, Mountain View, CA). Cells were then washed and resuspended in 1× PBS, 3% FCS, and 0.2% NaN_3, and analyzed using a FACStar Plus (Becton Dickinson, Bedford, MA).

Transduction of CD34$^+$ Cells. All transductions were performed at a final concentration of 5×10^4 cells/ml on fibronectin-coated plates (Collaborative Biomedical, Bedford, MA) in IMDM, 20% FCS, and 1% penicillin/streptomycin in the presence of 4 μg/ml polybrene over a 24-h period, with two changes of viral supernatant. Transductions with cell-free A12M1 MDR retroviral supernatants were performed either with or without a 24–48-h preincubation period and/or with or without growth factors, as described in individual experiments. The multiplicity of infection was maintained for all experiments at 2:1. The growth factors used were 50 ng/ml IL-3, 50 ng/ml IL-6, and 50 ng/ml stem cell factor (all gifts from Amgen, Thousands Oak, CA). At the end of this period, cells were taken for clonogenic assays.

Clonogenic Assays. PBSC CD34$^+$ cells were assayed for clonogenic activity by plating in methylcellulose, essentially as described previously (11, 12). Generally, 2×10^5 cells were diluted in 300 μl IMDM and 20% FCS and added to 3 ml Ready-Mix methylcellulose (Stem Cell Technologies, Vancouver, Canada). IL-3 and granulocyte-macrophage colony-stimulating factor were added to a final concentration of 10 units/ml. In some experiments, taxol (Bristol-Meyers Squibb, Princeton, NJ) was added to the mix at a final concentration of 10 ng/ml. The cell-methylcellulose mix was vortexed, and bubbles dissipated. One-half of the cell suspension was placed on each of two 35-mm gridded plates with a 3-ml syringe and 16-gauge needle. Methylcellulose was spread evenly over the dish, and the plates were incubated at 37°C in 5% CO_2. BFU-E and CFU-GM were scored after 15–18 days, and some were picked and analyzed as described below. The number of colonies obtained from each experiment for different culture conditions was normalized by plating the same number of cells for all experiments.

PCR Analysis. Individual methylcellulose colonies were aspirated by mouth pipette from the semisolid culture and washed in 1 ml PBS. The resulting cell pellet was lysed in 50 μl proteinase K buffer and then incubated at 95°C for 10 min to inactivate the proteinase K. PCR was carried out on 25 μl lysate with 1 unit AmpliTaq polymerase and the appropriate reaction mix (Perkin Elmer, Norwalk, CT) using 20 μM of the following *MDR* cDNA sequence primers: sense strand, CCCATCATTGCAATAGCAGC (residues 2596–2615); and antisense strand, GTTCAAACTTCTGCTCCTGA (residues 2733–2752), derived from two adjacent exons of the human *MDR* gene (8, 13). A 167-bp product, which is specific for the *MDR* cDNA and not the endogenous (intron-containing) *MDR* gene, was amplified with 35 cycles of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 60 s of extension at 72°C. Twenty μl of each reaction were run on 4% NuSieve (FMC Bioproducts, Rockland, ME) agarose gels. As a control for DNA content, 25 μl of each lysate were also analyzed using human β-globin-specific primers amplifying a 165-bp band (14).

Table 1 Effect of growth factors and preincubation

Experiment	Preincubation[a]	Growth factors	BFU-E[b]	CFU-GM[b]
1	+	+	10/10	8/10
2	+	+	3/20	5/20
3	+	+	13/25	4/19
4	+	+	6/10	7/10
5	+	+	17/20	13/15
6	+	+	19/20	14/15
Total			68/105	51/89
Total %			64.7	57.3
7	−	+	0/10	1/10
8	−	+	0/10	0/10
Total			0/20	1/20
Total %			0.0	5.0
9	+	−	3/10	3/10
10	+	−	2/25	7/15
Total			5/35	10/25
Total %			14.3	40.0
11	−	−	2/25	2/10
12	−	−	0/10	0/10
Total			2/35	2/20
Total %			5.7	10.0

[a] +, with; −, without.
[b] Number of PCR-positive colonies/number of colonies analyzed.

RESULTS

***MDR* Transduction of CD34$^+$ Cells from PBSCs.** CD34$^+$-selected PBSCs were 63.8 ± 20% positive by FACS analysis using an anti-CD34 antibody; there was approximately a 10–100-fold increase in BFU-E and a 12–325-fold increase in CFU-GM over the pre-CD34-selected cell population, as expected. Following A12M1 transduction, CD34$^+$ cells were positive for *MDR* gene transfer by *MDR* PCR analysis of cell suspensions directly after transduction (data not shown). The transduction efficiency was determined by analysis of individual methylcellulose colonies by PCR for the transferred *MDR* cDNA using *MDR* cDNA-specific primers (Fig. 1 and Table 1; Ref. 9). All untransduced samples treated similarly and analyzed identically in all experiments had no positive colonies by *MDR* PCR analysis. In six different experiments, using both preincu-

Table 2 Taxol resistance of clonogenic progenitors

	Untransduced taxol[a]			Transduced taxol[a]		
	−	+	% resistant[b]	−	+	% resistant[b]
Experiment 1						
BFU-E	339	5	1.5	423	95	22
CFU-GM	48	7	14	98	47	48
Experiment 2						
BFU-E	78	0	0	87	17	19
CFU-GM	56	0	0	86	18	20
Experiment 3						
BFU-E	120	3	2.5	124	32	26
CFU-GM	58	2	3.4	33	16	48

[a] Number of colonies/2 × 10^5 cells. −, without; +, with.
[b] Colonies with taxol/colonies without taxol.

bation and growth factors, 15–100% of the BFU-Es analyzed were positive, with a total of 68 (64.7%) of 105 positive overall (Table 1 and Fig. 1). *MDR* PCR-positive CFU-GM colonies in the six experiments ranged from 21 to 93%, with a total of 51 (57.3%) of 89 positive (Table 1, experiments 1–6).

Table 1 also shows a summary of transduction efficiencies comparing different protocols used. Omission of preincubation and/or growth factors lowered transduction efficiencies to as little as 0%, with a greater effect seen in the absence of preincubation prior to transduction than in the absence of growth factors (Table 1, experiments 7–12).

Taxol Resistance of Progenitor Colonies. In three experiments using the optimal conditions of 48-h preincubation and the presence of growth factors, taxol was added to the clonogenic assays to test for the functional expression of the transferred *MDR* gene. The number of colonies derived from the transduced and untransduced $CD34^+$ cells grown without taxol are comparable (Table 2). The cells used in experiments 1–3 in Tables 1 and 2 are identical; the percentage of *MDR* PCR-positive colonies derived from transduced $CD34^+$ cells without taxol in these three experiments is between 15 and 100%, as shown in Table 1. In each of the three experiments in Table 2, we show that 19–26% of BFU-E colonies and 20–48% of CFU-GMs from transduced $CD34^+$ cells are taxol resistant, compared with none to very few taxol-resistant colonies in untransduced cells (Table 2). In almost all cases, the percentages of *MDR* PCR-positive colonies in Table 1 are higher than the percentages of taxol-resistant clones in the same cell populations in Table 2. This is probably due to the fact that *MDR* expression from the transduced gene may not be sufficient to provide taxol resistance in all cases, depending presumably on the site of integration of the MDR retroviral vector. In only one case, CFU-GM in experiment 3, does the percentage of taxol resistance exceed the percentage *MDR* PCR-positive cells. This may be due to the fact that if inadequate amounts of DNA are available from individual clones, a negative *MDR* PCR may result, and the transduction efficiency may be underestimated. As expected, almost all of the individual BFU-Es and CFU-GMs resistant to taxol were *MDR* PCR positive (data not shown).

DISCUSSION

Our previous studies with $CD34^+$-selected BM progenitors demonstrated overall *MDR* gene transfer efficiencies of 39% in BFU-Es and 45% in CFU-GMs and showed an increased expression of MDR by FACS analysis (9). In two additional clonogenic experiments in methylcellulose with BM progenitors under the same optimal conditions used here, 40 and 72% of BFU-Es and 50 and 80% of CFU-GMs were *MDR* PCR positive.[5]

Optimal transduction efficiency in PBPCs requires both growth factors and preincubation (Table 1, experiments 1–6). In this study, we show at least as efficient transfer of the *MDR* gene into the hematopoietic progenitors of $CD34^+$-enriched PBPCs as we reported previously in BM cells. Up to 100% of BFU-Es from transduced $CD34^+$ cells in one experiment contain the transferred *MDR* cDNA. Others have reported similar transduction efficiencies using the neomycin gene into marrow- and PBPC-derived $CD34^+$ cells (15). With a significant range of variations from patient to patient, we conclude that the transduction efficiencies of BM- and PBSC-derived human hematopoietic progenitors seem comparable.

Retroviral gene transfer is less efficient without a preincubation period and/or without growth factors (Table 1). This decrease is seen to a lesser extent when growth factors are omitted compared with no preincubation. In the absence of both preincubation and growth factors, *MDR* transduction was also very low (Table 1, experiments 11 and 12). In murine marrow, it has been consistently shown that preincubation for 48 h with IL-3 and IL-6 (16, 17) or IL-6 and stem cell factor (18) improves gene transfer efficiency, as analyzed *in vivo*. The effect of growth factor exposure and preincubation on transduction efficiency is most likely due to the need for cells to be actively cycling for retroviral gene integration. In contrast to BM progenitors, we thought that perhaps a different biological state of activation by *in vivo* stimulation of PBPCs by growth factors and chemotherapy prior to apheresis might recruit PBPCs into the cell cycle and, therefore, negate the need for growth factors and preincubation. However, in this analysis, we show that the most efficient retroviral transduction protocol includes a preincubation period in the presence of growth factors.

The requirement for preincubation seems greater than the need for exogenous growth factors (Table 1). The lack of preincubation probably does not allow cell replication and may prevent cell survival as well over 48 h. A period of preincuba-

[5] M. Ward, P. Pioli, C. Hesdorffer, and A. Bank, unpublished observations.

tion even without growth factors (Table 1, experiments 9 and 10) leads to greater transduction efficiency than no preincubation (Table 2, experiments 7, 8, 11, and 12). This may be due to: (*a*) the need for *in vitro*-conditioning of cells after harvest for their integrity; and/or (*b*) the presence of factors in the FCS other than the added growth factors that optimize their transduction. Clearly, both preincubation and the presence of growth factors are required for optimal transduction (Table 1).

It has been suggested that stroma is required for optimal hematopoietic stem cell transduction (19). We have, however, obtained transduction efficiencies equal to or exceeding those reported by those investigators. This may be due to our use of fibronectin plates for $CD34^+$ cell culture, which may mimic the effect of stroma, at least partially.

We have previously shown long-term *MDR* gene transfer and expression of hematopoietic progenitors in mice, indicating transduction of murine hematopoietic stem cells (8, 20). We have also demonstrated transduction of marrow-derived $CD34^+$ cells using long term colony-initiating assays (9). However, only *in vivo* studies of transduced cells in humans will definitively show the efficiency of long-term human hematopoietic stem cell transduction and expression.

The ultimate goal of *MDR* gene transfer into hematopoietic stem cells is to produce a resistant population of marrow cells that is protected against the myelosupressive effects of chemotherapeutic agents. In this study, we provide evidence that this is the case with our findings that clonogenic progenitors from *MDR*-transduced $CD34^+$ PBPCs are resistant to taxol (Table 2). This progenitor population of chemotherapy-resistant cells could potentially be further selected for, either by FACS cell sorting *in vitro* or *in vivo* in patients undergoing cancer chemotherapy following autologous BM transplantation. The ability to selectively expand *MDR*-expressing cells has already been shown in mice (7, 8). Another interesting application of the use of *MDR*-transduced cells is in combination with a nonselectable gene, such as globin, to enrich for transduced cells and, thus, potentially to compensate for the low gene transfer efficiencies (21–24).

REFERENCES

1. Kessinger, A., and Armitage, J. O. The evolving role of autologous peripheral stem cell transplantation following high-dose therapy for malignancies (Editorial). Blood, *77:* 211–213, 1991.

2. Duhrsen, U., Villeval, J., Boyd, J., Kannourakis, G., Morstyn, G., and Metcalf, D. Effects of recombinant human granulocyte colony stimulating factor on hematopoietic progenitor cells in cancer patients. Blood, *72:* 2074–2081, 1988.

3. Siena, S., Bregni, M., Brando, B., Bonadonna, G., and Gianni, A. M. Circulation of CD34+ hematopoietic stem cells in the peripheral blood of high-dose cyclophosphamide treated patients: enhancement by intravenous recombinant GM-CSF. Blood, *74:* 1905–1914, 1989.

4. Haas, R., Mohle, R., Fruehauf, S., Goldschmidt, H., Witt, B., Flentje, M., Wannenmacher, M., and Hunstein, W. Patient characteristics associated with successful mobilizing and autografting of peripheral blood progenitors (PBPC) in malignant lymphoma. Blood, *83:* 3787–3794, 1994.

5. Pastan, I., and Gottesman, M. M. Multidrug resistance. Annu. Rev. Med., *42:* 277–286, 1991.

6. Chaudhary, P. M., and Roninson, I. B. Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell, *66:* 85–94, 1991.

7. Sorrentino, B. P., Brandt, S. J., Bodino, G., Gottesman, M., Pastan, I., Cline, A., and Nienhuis, A. W. Selection of drug-resistant bone marrow cells *in vivo* after retroviral transfer of human *MDR1*. Science, *257:* 99–103, 1992.

8. Podda, S., Ward, M., Himelstein, A., Richardson, C., de la Flor-Weiss, E., Smith, L., Gottesman, M., Pastan, I., and Bank, A. Transfer and expression of the human multiple drug resistance gene into live mice. Proc. Natl. Acad. Sci. USA, *89:* 9676–9680, 1992.

9. Ward, M., Richardson, C., Pioli, P., Smith, L., Podda, S., Goff, S., Hesdorffer, C., and Bank, A. Transfer and expression of the human multiple drug resistance gene in human CD34+ cells. Blood, *84:* 1408–1414, 1994.

10. Berenson, R. J., Bensinger, W. I., Kalamasz, D. F., Heimfeld, S., Goffe, R. A., Berninger, R. W., Peterson, D. R., Thompson, P., and Strong, D. M. Transplantation of stem cells enriched by immunoadsorption. *In:* Advances in Bone Marrow Purging and Processing, pp. 449–459. New York: Wiley-Liss, Inc., 1994.

11. Sutherland, H., Eaves, A., and Eaves, C. Quantitative assays for human hematopoietic progenitor cells. *In:* A. Gee (ed.), Bone Marrow Processing and Purging: A Practical Guide, pp. 155–171. Boca Raton, FL: CRC Press, Inc., 1994.

12. Sutherland, H. J., Eaves, C. J., Eaves, A. C., Dragowska, W., and Lansdorp, P. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis *in vitro*. Blood, *74:* 1563–1570, 1989.

13. Noonan, K. E., Beck, C., Holzmayer, T. A., Chin, J. E., Wunder, J. S., Andrulis, I. L., Gazdar, A. F., Willman, C. L., Griffith, B., Von Hoff, D. D., and Roninson, I. B. Quantitative analysis of *MDR1* (multidrug resistance) gene expression in human tumors by polymerase chain reaction. Proc. Natl. Acad. Sci. USA, *87:* 7160–7164, 1990.

14. Kan, Y. W., and Chehab, F. F. Detection of specific DNA sequences by fluorescence amplification: a color complementation assay. Proc. Natl. Acad. Sci. USA, *86:* 9178–9182, 1989.

15. Cassel, A., Cottler-Fox, M., Doren, S., and Dunbar, C. Retroviral-mediated gene transfer into CD34-enriched human peripheral blood stem cells. Exp. Hematol., *21:* 585–591, 1993.

16. Bodine, D. M., Karlsson, S., and Nienhuis, A. W. Combination of interleukins 3 and 6 preserves stem cell function in culture and enhances retrovirus-mediated gene transfer into hematopoietic stem cells. Proc. Natl. Acad. Sci. USA, *86:* 8897–8901, 1989.

17. Apperley, J. F., Luskey, B. D., and Williams, D. A. Retroviral gene transfer of human adenosine deaminase in murine hematopoietic cells: effect of selectable marker sequences on long-term expression. Blood, *78:* 310–317, 1991.

18. Luskey, B. D., Rosenblatt, M., Zsebo, K., and Williams, D. A. Stem cell factor, interleukin-3, and interleukin-6 promote retroviral-mediated gene transfer into murine hematopoietic stem cells. Blood, *80:* 396–402, 1992.

19. Nolta, J., Smogorzewska, E., and Kohn, D. Analysis of optimal conditions for retroviral-mediated transduction of primitive human hematopoietic cells. Blood, *86:* 101–110, 1995.

20. Richardson, C., and Bank, A. Preselection of transduced murine hematopoietic stem cell populations leads to increased long-term stability and expression of the human multiple drug resistance gene. Blood, *86:* 2579–2589, 1995.

21. Aran, J., Gottesman, M., and Pastan, I. Drug-selected coexpression of human glucocerebrosidase and P-glycoprotein using a bicistronic vector. Proc. Natl. Acad. Sci. USA, *91:* 3176–3180, 1994.

22. Germann, U., Gottesman, M., and Pastan, I. Expression of a multidrug resistance adenosine deaminase fusion gene. J. Biol. Chem., *264:* 7418–7424, 1989.

23. Kane, S., Reinhaud, D., Fordis, C., Pastan, I., and Gottesman, M. A new vector using human multiple drug resistance gene as a selectable marker enables overexpression of foreign genes in eukaryotic cells. Gene (Amst.), *84:* 439–446, 1989.

24. Kane, S., Truer, B., Gal, S., Ueda, K., Pastan, I., and Gottesman, M. Use of a cloned multidrug resistance gene for co-amplification and overproduction of MFP, a transformation regulated secreted acid protease. Mol. Cell. Biol., *8:* 439–446, 1989.

Clinical Cancer Research

Retroviral transfer and expression of the human multiple drug resistance (MDR) gene in peripheral blood progenitor cells.

M Ward, P Pioli, J Ayello, et al.

Clin Cancer Res 1996;2:873-876.