Gene Transfer Into Hematopoietic Progenitor Cells From Normal and Cyclic Hematopoietic Dogs Using Retroviral Vectors

By Martin A. Eglitis, Phillip W. Kantoff, Janet D. Jolly, J.B. Jones, W. French Anderson, and Clinton D. Lothrop, Jr

The Moloney murine leukemia retrovirus-derived vector N2 was used to transfer the bacterial NeoR gene (confering resistance to the neomycin analogue G418) into hematopoietic progenitor cells. Approximately 5% of day seven CFU-GM were resistant to 2,000 μg/ml G418, using a supernatant infection protocol in the absence of vector-producing cells. A greater proportion of CFU-GM colonies were recovered relative to uninfected controls as the stringency of selection was diminished. Enzyme activity was detected in drug-resistant colonies, confirming that the resistant colonies obtained after infection with N2 represented cells producing neomycin phosphotransferase. Activity in the CFU-GM colonies approached 50% of that of drug-resistant vector-producing cells on a per cell basis. To test the hypothesis that more rapidly cycling bone marrow cells would be more susceptible to vector infection, we treated progenitor cells obtained from cyclic hematopoietic (CH) dogs with the N2 vector. Despite the increased numbers of hematopoietic progenitor cells obtained from CH dogs, the proportion of G418-resistant CFU-GM did not increase over that obtained with N2-infected normal marrow. These results demonstrate that retroviral vectors can be used to transfer and express exogenous genes in canine hematopoietic progenitor cells.

THE DEVELOPMENT of retroviral-based genetic vectors has provided a means to introduce foreign genes efficiently into hematopoietic stem cells. In addition to their utility for studying questions of gene regulation in hematopoietic tissue, retroviral vectors may potentially be used for gene therapy of hematopoietic cells. The bone marrow is a likely initial target for gene therapy because extensive experience exists in manipulating and treating this tissue ex vivo and because some genetic diseases exert a primary tissue effect in the marrow.

Several groups have reported successful transfer and expression of genes into bone marrow cells of a variety of animals in vitro. The degree of success as measured by the proportion of drug-resistant colonies generated in vitro differs between species and is generally highest in mice. Whether these species differences correspond to differences in the absolute level of enzyme expression is unclear. Even more dramatic species differences have been seen in the in vivo level of expression between mice, non-human primates, and fetal sheep.

The dog represents an attractive model for autologous bone marrow transplantation combined with retrovirus-mediated gene transfer. A canine model for bone marrow transplantation has been described. Furthermore, canine models of hemophilia A and B, hemolytic anemia due to pyruvate kinase deficiency or phosphofructokinase deficiency, a lysosomal storage disease model due to α-iduronidase deficiency are all well-characterized animal models of the human disorders and potential models for gene therapy. Toward the ultimate goal of establishing an in vivo gene transfer model in dogs, we report our initial results on gene transfer in canine hematopoietic stem cells in vitro. Our observations confirm, in part, the initial report of Kwok et al that canine bone marrow cells are infectable by retroviral vectors. In contrast, however, we find infectability only with a supernatant not a cocultivation protocol. Furthermore, in addition to finding 3% to 5% G418-resistant CFU-GM, we demonstrated the presence of neomycin phosphotransferase (generated from the NeoR gene) in CFU-GM colonies and showed that progenitor cells from cyclic hematopoietic (CH) dogs are infectable to no greater extent than are normal bone marrow cells.

MATERIALS AND METHODS

Animals. Normal dogs were young adults of both sexes and were in apparent good health. Five cyclic hematopoietic dogs (three females, two males) ranging in age from 4 to 18 months were used in these studies. The colony of CH dogs has been previously described. Bone marrow was obtained by percutaneous aspiration with pentobarbital sedation. All dogs were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

Colony assays. Canine CFU-GM were determined as previously described. In brief, 7.5 × 105 nucleated bone marrow cells were plated in 1 mL Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) containing 15% fetal calf serum (FCS) (Hyclone, Logan, UT), 10% newborn dog serum in either 0.36% agar or 0.8% methylcellulose. The antibiotic G418 (GIBCO), when added, was present in the CFU-GM assay at concentrations of 250 to 2,000 μg/mL as indicated. The bone marrow cells were cultured at 37 °C in a 5% CO2 atmosphere, and the CFU-GM were scored at 5 to 8 days.

Vector for gene transfer. The retroviral vector N2, which has been previously described in detail, was used to characterize the infectability of canine bone marrow cells. This vector, derived from the Moloney murine leukemia virus, contains the bacterial NeoR gene, which confers resistance to the neomycin analogue G418 in eukaryotic cells. The N2 vector was introduced into the amphotropic packaging cell line PA12 by conventional calcium phosphate transfection. G418-resistant clones were isolated and one, V6, with

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a titer of 3 to 5 x 10⁴ drug-resistant colony-forming units (CFUs)/ml. was used for these studies. This clone (V6) contained -0.1% helper virus contamination; however, the integrity of the N2 vector was unaffected.

Infection procedures. Bone marrow progenitor cells were infected by two general protocols. In the first, 1 x 10⁸ canine marrow cells were cocultivated with an ~80% confluent V-6 monolayer that had been irradiated two hours previously with 15 Gy from a ¹³⁷Cs source. During cocultivation, polybrene was added to a final concentration of 4 µg/mL. After an 18-hour infection, the nonadherent marrow cells were recovered by rinsing the tissue culture flask (75 cm²) with IMDM. The recovered cells were washed three times with 10 mL IMDM, pelleted by low-speed centrifugation, and plated in the CFU-GM colony assay.

Alternatively, bone marrow cells were exposed to vector-containing medium without fibroblast producer cells. In this “supernatant” protocol, 3.7 x 10⁶ nucleated bone marrow cells were mixed with 7.5 mL V-6-conditioned medium. Polybrene (in the amount indicated herein) was added, and the suspension was incubated for four hours with gentle mixing every 15 minutes. After infection, the bone marrow cells were recovered by low-speed centrifugation, washed three times with 10 mL complete medium, and plated in the CFU-GM assay. At the time of plating, bone marrow cell viability was >95% with either infection protocol.

Enzyme analyses. Neomycin phosphotransferase (NPT) was assayed according to the method of Reiss et al. Cells were recovered from methylcellulose cultures, washed extensively, and then lysed by five freeze/thaw cycles. The cleared lysate was separated using nondenaturing polyacrylamide gel electrophoresis (PAGE), and then enzyme activity was measured as described previously.

Statistical methods. Statistical significance was determined with Student’s t test. All data are presented as mean ± SEM.

RESULTS

Canine hematopoietic progenitor cells cultured in soft agar or methylcellulose produced 10.9 ± 10.2 CFU-GM/7.5 x 10⁶ cells. Addition of the neomycin analog G418 to the culture substantially reduced the number of uninfected control CFU-GM recovered at a dose of 2,000 µg/mL (Table 1). Cocultivation of bone marrow progenitor cells with vector-producing fibroblast cells did not result in G418-resistant CFU-GM (Table 1), even though essentially 100% of progenitor cells from such an infection were recovered as viable CFU-GM if plated without G418. Similarly, incubation of canine bone marrow cells with PA12 cells prior to plating in the CFU-GM assay did not inhibit the CFU-GM colonies (data not shown). In contrast, a low but significant (P < .005) number of G418-resistant colonies were recovered after the supernatant infection protocol (Table 1). Approximately 5% of CFU-GM were drug resistant after a four-hour infection with viral-containing supernatant. This level of infection was apparently not due to limiting amounts of virus, since dilution of viral supernatant by 1:10 resulted in about the same proportion of G418 resistant CFU-GM (Table 1). Incubation of bone marrow cells with N2-rich supernatant without subsequent G418 selection showed that no CFU-GM were lost by this treatment (data not shown).

Presence of NPT in colonies. To confirm that the resistant colonies obtained after infection with N2 represented cells producing NPT, pools of drug-resistant colonies were recovered and assayed for enzyme activity. A representative result is shown in Fig 1: G418-resistant CFU-GM obtained from two independent infections were analyzed for NPT activity. Arrow indicates the expected location of the 29-kd NPT protein. Lanes, 1, 2, and 4 show the activity found in pools of ~50 CFU-GM (1.7 x 10⁶, 1.4 x 10⁶, and 6.5 x 10⁵ cells, respectively) resistant to 2,000 µg/mL G418 after infection with N2. NPT activity was not found in normal, uninfected CFU-GM (lane 3, 1.5 x 10⁶ cells loaded).

Effect of G418 concentration on recovery of drug-resistant CFU-GM. The correlation between detectable NPT enzyme activity and drug-resistant CFU-GM is not clear. Because the initial selection conditions of 2,000 µg G418/mL may have been too stringent, the difference in numbers of CFU-GM recovered between uninfected and infected marrow at various concentrations of G418 were compared. The G418-resistant CFU-GM increased both with and without infection as the G418 concentration was decreased (Table 2), but the difference in colonies recovered between vector-infected and vector-uninfected marrow cultures increased as the level of G418 used for selection was decreased (Fig 2).

Analysis of marrow from CH dogs. The ability of retroviruses to infect cells productively requires that target cells be actively dividing. The proportion of actively dividing progenitor cells in bone marrow is small, which might contribute to the low proportion of infected CFU-GM. To analyze cell cycling effects on retroviral-mediated gene transfer in hematopoietic progenitor cells, we used a canine model for cyclic hematopoiesis. In the CH dogs, generation of hematopoietic cells is not continuous; rather, the cells of the hematopoietic system are generated in waves. Therefore, progenitor cells undergo periods of proliferation at predictable intervals. We reasoned that hematopoietic cells from the CH dogs might be particularly amenable to vector infection because of the presence of large proportions of cycling cells. A 50% increase in the number of CFU-GM was

<table>
<thead>
<tr>
<th>Table 1. Co-cultivation v Supernatant Infection Protocols</th>
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<tbody>
<tr>
<td>Bone Marrow Treatment</td>
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<tr>
<td>-----------------------</td>
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<tr>
<td>G418-resistant CFU-GM (%)</td>
</tr>
</tbody>
</table>

Percentages are mean ± SEM of results obtained from 12 independent infections. In any given experiment, at least 400 CFU-GM were plated to determine percentage of G418-resistant cells. Selection was in 2,000 µg/mL G418. The percentage of G418-resistant CFU-GM was significantly greater (P < .005) in the supernatant-infected and 1:10 diluted supernatant-infected groups but not the co-cultivation group (P > .05) relative to the uninfected control group.
Fig 1. Neomycin phosphotransferase activity in pools of G418-resistant canine CFU-GM. Colonies resistant to 2,000 μg/mL G418 were recovered from methylcellulose culture, washed extensively, and assayed. Lanes 1 and 2 show activity in two different pools of cells from the same infection. Lane 3 shows activity in a pool of uninfected, unselected CFU-GM. Lane 4 shows activity in a pool of CFU-GM from a second infection experiment. Arrow indicates position of expected 29-kd NPT protein. Numbers of cells in each pool are as follows: lane 1, 1.7 x 10⁶; lane 2, 1.4 x 10⁶; lane 3, 1.5 x 10⁶; lane 4, 6.5 x 10⁶.

seen in CH dogs on the second to fourth days of their cycle as compared with normal dogs (Table 2). The sensitivity of uninfected CH progenitors to G418 was roughly comparable to that of uninfected progenitors from normal dogs. A significant (P < .01) number of G418-resistant CFU-GM were recovered after infection of CH progenitor cells relative to control uninfected CH progenitor cells (Table 2). Nonetheless, cells from CH dogs at the time of maximal hematopoiesis were no more infectable (P > .1) with the N2 vector than marrow from normal control dogs infected in parallel experiments. Administration of cytoxan to a normal dog elicited a similar burst of hematopoietic proliferation that also did not result in increased infection of hematopoietic progenitors (data not shown).

Infection variables. Because a greater number of G418-resistant CFU-GM were observed with a lower drug concentration, we determined whether increasing the interval between infection and actual selection would increase the number of G418-resistant CFU-GM by allowing infected cells more time to express the transferred NeoR gene. Bone marrow cells were infected for four hours, using the supernatant protocol, and were immediately plated in selective medium or allowed to rest overnight in liquid culture without

Fig 2. Difference in G418-resistant CFU-GM between N2 vector-infected and vector-uninfected canine bone marrow at increasing concentrations of G418. The difference in G418-resistant CFU-GM was calculated from the mean number of G418-resistant CFU-GM summarized in Table 2 and contains a similar range of experimental error.

Table 2. G418-Resistant CFU-GM Before and After Infection With the N2 Vector

<table>
<thead>
<tr>
<th>G418 Concentration (μg/mL)</th>
<th>Normal Control—Uninfected</th>
<th>Normal Infected</th>
<th>CH Control Uninfected</th>
<th>CH Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>109.0 ± 10.2 (24)</td>
<td></td>
<td>172.0 ± 103.4 (8)</td>
<td>ND</td>
</tr>
<tr>
<td>125</td>
<td>48.0 ± 13.3 (4)</td>
<td>81 (1)</td>
<td>96 (1)</td>
<td>ND</td>
</tr>
<tr>
<td>250</td>
<td>66.8 ± 14.6 (6)</td>
<td>104 (1)</td>
<td>56.3 ± 22.5 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>500</td>
<td>47.9 ± 17.2 (6)</td>
<td>109 (1)</td>
<td>31.7 ± 19.6 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>1,000</td>
<td>18.4 ± 6.7 (7)</td>
<td>39.6 ± 7.0 (3)</td>
<td>8.8 ± 5.2 (3)</td>
<td>ND</td>
</tr>
<tr>
<td>1,500</td>
<td>2.0 ± 1.3 (9)</td>
<td>7.9 ± 3.4 (8)</td>
<td>0.1 ± 0.1 (3)</td>
<td>1.5 ± 0.4 (2)</td>
</tr>
<tr>
<td>1,750</td>
<td>2.0 ± 0.8 (9)</td>
<td>5.0 ± 1.0 (12)</td>
<td>2.7 ± 2.7 (4)</td>
<td>4.7 ± 1.9 (4)</td>
</tr>
<tr>
<td>2,000</td>
<td>0.8 ± 0.3 (16)</td>
<td>3.2 ± 0.6 (15)</td>
<td>0.0 ± 0.0 (6)</td>
<td>3.2 ± 1.2 (4)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM; ND, not done. Numbers in parentheses indicate number of platings. At G418 concentration of 1,000 μg/mL, uninfected v infected normals differed at a significance of P = .05. The significance of the difference in normal subjects at G418 concentrations of 1,500, 1,750, and 2,000 μg/mL are P < .075, 0.02, and 0.005, respectively. Similarly, the significance of the difference in CH CFU-GM is P < .1, P > .1 and P < .005 for 1,500, 1,750, and 2,000 μg G418/mL. The percentage of G418-resistant of normal subjects v CH after infection was not significantly different (P > .1 in each case).
of CFU-GM used for selection, similar results were found once total numbers of colonies were normalized to the uninfected background. Lower levels of drug resistant CFU-GM were found at polybrene concentrations of 2, 4, and 40 μg/mL than at concentrations of 8 or 16 μg/mL. The combination of greatest total recovery and highest proportion of resistant CFU-GM was obtained after infection in the presence of 8 μg/mL, with ~5% of CFU-GM resistant to G418.

**DISCUSSION**

Retroviral vectors provide an efficient means for the transfer of an exogenous gene into hematopoietic cells in vitro.5,6 We confirmed the results of Kwok et al.6 that a gene can be transferred into canine CFU-GM with an efficiency similar to that reported in vitro for mouse,9 human,3,5 and monkey14 CFU-GM. Such results point to the potential for gene therapy with retroviral vectors. We have begun to characterize retroviral-mediated gene transfer in canine hematopoietic progenitor cells in vitro as one step in the development of a large animal model for gene therapy.

In vitro colony assays are a sensitive method to detect expression of transferred drug-resistance genes. Direct assay of the enzyme is necessary, however, for accurate measurement of gene expression. In pools of G418-resistant CFU-GM, we demonstrated NPT activity after three independent infections (Fig 1; data not shown). In all cases, the level of NPT activity was at least 10% that of a G418-resistant virus producer cell line on a per cell basis and, in one case, NPT activity was >50% of a virus producer line (Fig 1, lane 2). These results indicate that drug resistance corresponds to measurable levels of NPT. In no case, however, is the expression as great as that found in NIH-3T3 cells. Whether this lower level of expression is due to a decrease in the transcriptional activity of the viral promoter in canine hematopoietic cells is unclear.

In an effort to determine if infection conditions for dog marrow cells could be optimized, we analyzed a number of variables. In our hands, drug-resistant CFU-GM could only be after infection with viral medium in the absence of vector-producing cells. This result contrasts with that of

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**Table 3. Effect of Preselective Rest on Recovery of G418-Resistant CFU-GM**

<table>
<thead>
<tr>
<th>G418 Concentration (μg/mL)</th>
<th>Period of Rest After Infection</th>
<th>Percentage of G418-Resistant CFU-GM*</th>
<th>Viability†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hour</td>
<td>18 hour</td>
<td></td>
</tr>
<tr>
<td>1.75</td>
<td>3.0 ± 0.5*</td>
<td>1.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>2.000</td>
<td>2.2 ± 1.2</td>
<td>1.7 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>

*Percentage of G418-resistant CFU-GM ± SEM.

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**Table 4. Polybrene Effects**

<table>
<thead>
<tr>
<th>Polybrene Concentration (μg/mL)</th>
<th>Percentage of CFU-GM Recovered*</th>
<th>Percentage of Viability†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>123 (1)</td>
<td>91.3 ± 8.7 (3)</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td>121.3 ± 36.7 (2)</td>
</tr>
<tr>
<td>4</td>
<td>117.2 ± 11.1 (5)</td>
<td>113.7 ± 27.2 (7)</td>
</tr>
<tr>
<td>8</td>
<td>92.5 ± 2.5 (2)</td>
<td>95.9 ± 39.7 (5)</td>
</tr>
<tr>
<td>12</td>
<td>89 (1)</td>
<td>86.9 ± 39.9 (2)</td>
</tr>
<tr>
<td>16</td>
<td>65.4 ± 5.6 (2)</td>
<td>72.3 ± 11.3 (2)</td>
</tr>
<tr>
<td>40</td>
<td>66.4 ± 33.0 (3)</td>
<td>56.2 ± 4.9 (2)</td>
</tr>
</tbody>
</table>

*Values are percentages of untreated CFU-GM ± SEM; 4-hour exp.; 4 hour treatment at the indicated concentration of polybrene before plating in the CFU-GM assay without G418; 0/N Exp., overnight (18 hour) treatment in the indicated concentration of polybrene before plating in the CFU-GM assay without G418; n, number of experiments; ND, not done.

†Percentage of viability was measured after an overnight (18 hour) exposure to the indicated concentration of polybrene. Viability was determined by trypan blue exclusion.

‡Percentage of G418-resistant CFU-GM was determined with the supernatant infection protocol as described.
Kwok et al, who obtained drug-resistant CFU-GM only after a cocultivation infection protocol. The reason for the failure of the cocultivation infection is unclear, but apparently cocultivation per se was not toxic since there was no decrease in the absolute proportion of CFU-GM recovered. The cell line we used for infections is essentially equivalent to the PA12-N2 of Kwok et al so that cell effects are not a likely explanation. Although the V6 cell line was contaminated with helper virus, studies with human bone marrow cells indicate that such contamination does not affect the observed infection rate (M.A. Eglitis et al, unpublished observations, 1987).

Because the production of NPT was less in canine hematopoietic cells than in N2-containing NIH-3T3 cells, the concentration of G418 used for selection was decreased to determine if greater numbers of drug-resistant CFU-GM could be recovered. Decreasing the G418 concentration from 2,000 to 1,000 \( \mu \text{g/mL} \) increased the difference between infected and uninfected CFU-GM resistant as a percentage of unselected controls from 2.4 to 21.2\% (Table 2 and Fig 2). Thus, vector-infected CFU-GM apparently express a spectrum of NPT activities so that selection with a lower concentration of drug permitted a greater proportion of CFU-GM to survive.

Bone marrow cells obtained from dogs with CH at the time of peripheral neutropenia when the bone marrow is presumably enriched for rapidly cycling pools of progenitor cells based on tritium suicide data also had no increased infection efficiency. Although there is some discrepancy as to occurrence of cycles of tritium-sensitive CFU-GM in CH dogs, clear evidence shows a two- to fivefold increase in tritium sensitivity of bone marrow CFU-GM during the peripheral neutropenia on days one to four (Table 2 and Fig 2). Thus, vector-infected CFU-GM apparently express a spectrum of NPT activities so that selection with a lower concentration of drug permitted a greater proportion of CFU-GM to survive.

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Other rapidly cycling hematopoietic progenitor cells have been studied for their infectivity with retroviral vectors. Hogge and Humphries tested the infectivity of hematopoietic progenitors obtained either from normal human fetal liver or from patients with chronic myelogenous leukemia. They also found that apparent increases in cell division rate (and/or a greater proportion of progenitors) did not appreciably improve progenitor cell infection rates in those human cells in vitro.

To determine if increasing the length of expression time of the neo \(^{\text{a}} \) gene could improve the treated cells' ability to survive incubation in G418, bone marrow cells were incubated in IMDM for 18 hours prior to G418 selection. There was no increase in the proportion of CFU-GM resistant to G418 after the 18-hour postinfection rest period. Polybrene concentrations used during exposure to virus were changed in an effort to maximize infection while minimizing toxicity. Although a distinct peak of optimal polybrene concentration was determined, this optimum still resulted in only \( \sim 5\% \) of CFU-GM being G418 resistant.

Several factors may be contributing to the inability to improve the level of infection of canine CFU-GM. For instance, their rarity may make progenitor cells inaccessible to the retroviral vectors. Another explanation may be that an intermediate efficiency at each of the multiple steps involved (ie, binding, uptake, integration, and expression) might combine to yield productive infection of no more than 10\% of canine CFU-GM. For example, binding affinity of the amphotropic envelope to receptors on canine cells could be too low to permit greater proportions of G418-resistant CFU-GM, or only the minority of integration sites may allow sufficient expression of the Neo \(^{\text{a}} \) gene to permit survival at toxic levels of G418. That expression may be a problem in recovering viable colonies when using drug selection is suggested by greater recovery of G418-resistant CFU-GM as the concentration of drug is lowered.

In vitro colony assays provide an efficient means of screening infection conditions to produce an optimal gene transfer protocol. The number of G418-resistant CFU-GM recoverable, however, are not always predictive of the proportion of bone marrow cells carrying the vector in vivo. For example, up to 20\% of monkey CFU-GM have been infected in vitro, whereas \(< 1\% \) of total marrow in the same monkeys contained and expressed vector genes in vivo. This discrepancy is due at least in part to the fact that different pools of cells are being assayed in vitro and in vivo. Therefore, the ultimate test of any infection protocol, no matter how efficient at infecting CFU-GM in vitro, is an analysis for the recovery of vector-containing marrow in vivo.

We are currently trying to extend these results to in vivo canine models for autologous bone marrow transplant/gene transfer. Canine models of human genetic diseases should be useful for characterizing the benefits and risks of gene therapy and for understanding how maximal expression of retroviral-transferred genes is best achieved.

REFERENCES

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Gene transfer into hematopoietic progenitor cells from normal and cyclic hematopoietic dogs using retroviral vectors

MA Eglitis, PW Kantoff, JD Jolly, JB Jones, WF Anderson and CD Jr Lothrop