Scaffold Attachment Region-Containing Retrovirus Vectors Improve Long-Term Proviral Expression after Transplantation of GFP-modified CD34+ Baboon Repopulating Cells.

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Abstract

Sustained high-level proviral expression is important for clinical applications of gene therapy. Genetic elements including the β-interferon scaffold attachment region (SAR) have been shown to improve transgene expression in hematopoietic cells. We hypothesized that SAR elements might improve expression and allow the preselection of successfully transduced cells. Thus, we transplanted three animals with GFP selected cells, half of which had been transduced with either SAR, or non SAR-containing retrovirus vectors. All animals showed delayed engraftment compared to historic controls (28 vs 15.5 days). GFP marking was seen at levels up to 8%, but declined over the first 6 weeks. Importantly, fluorescence intensity was 2-9-fold increased in progeny of SAR versus non SAR vector-modified cells in all hematopoietic lineages for the duration of follow-up (6-12 months). In conclusion, the use of SAR-containing vectors improved transgene expression which may obviate the need for multi-copy integration to achieve high-level expression and reduce the risk for insertional mutagenesis.
Introduction

Attenuation of proviral expression is a frequent occurrence after onco retroviral transduction of hematopoietic cells. Modifications in the design of the Moloney murine leukemia virus (MoMuLV) promoter/enhancer can improve transgene expression in the murine model\textsuperscript{1,2}, but not necessarily in the non human primate model\textsuperscript{3}. Alternative strategies to improve long-term expression in hematopoietic cells include the use of transcriptional modifiers such as SAR and insulator elements, or other post transcriptional regulatory sequences\textsuperscript{4-6}. SAR elements constitute \textit{cis}-regulatory elements that appear to create independent domains of transcription\textsuperscript{7}. Retrovirus vectors containing the SAR element of the human beta interferon (\(\beta\)-IFN) gene enhance the expression of heterologous reporter genes in T-cells, macrophages, and thymocytes \textit{in vitro} and \textit{in vivo}\textsuperscript{5,8-10}. The current study investigates if the presence of the \(\beta\)-IFN SAR element in an onco-retrovirus vector improves proviral GFP fluorescence in a large animal model and if these improvements may permit preinfusion selection of genetically modified hematopoietic cells to enhance post transplant chimerism. The latter strategy has been used successfully in murine stem cell gene transfer models\textsuperscript{11-13}, but so far not with success in a large animal model\textsuperscript{14}. 
Materials and Methods

Animals. Baboons (Papio cynocephalus cynocephalus or Papio cynocephalus anubis) were housed and transplanted under approved conditions as previously described \(^{15,16}\).

Retrovirus vectors. The retrovirus vectors encode the enhanced green fluorescent protein (EGFP, Clontech, Palo Alto CA) or its yellow variant, EYFP, under the control of the 5’ modified viral LTR \(^2\) and are otherwise identical. EGFP and EYFP genes were inserted into the MND-X retroviral vector plasmid \(^{17}\) to make the vectors MND-EGFP or MND-EYFP. The 800 bp human beta-IFN SAR element from J. Bode \(^7\) was inserted between the EGFP or EYFP gene and the 3’ LTR to make MND-EGFP-SAR or MND-EYFP-SAR. Producer cell clones with similar titer (0.6 – 1.3 x10\(^5\) HT1080 TU/ml) were generated using Phoenix GALV producer cells as described \(^{18}\).

CD34-enrichment, gene transfer and selection of GFP positive baboon marrow cells. Following 48 hour prestimulation CD34-enriched cells (generated as previously \(^{16}\)) were transduced twice for 4 hours in the presence of CH-296 (2 µg/cm RetroNectin\(^\text{TM}\): Takara Shuzo, Japan) \(^{16}\), Interleukin (IL) -3, -6, rhSCF, rhG-CSF, flt3-Ligand (Flt3-L), and megakaryocyte growth and development factor (MGDF) at 100 ng/ml. Cells (10 x 10\(^6\)) were exposed to a total of 60 ml volume of vector per flask resulting in a MOI between 0.1 and 0.3 in all three animals. Cells were sorted by flow-cytometry 36 hours after the last vector exposure according to EGFP or EYFP expression as previously described \(^3\).

Real-time PCR assay. For PCR amplification of provirus, DNA (300 ng) was amplified in duplicate with sequence specific primers and probes designed using Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, Calif.). EGFP: 5’- CTG CAC CAC CGG CAA -3’ and 5’- GTA GCG GCC GAA GCA CT -3’, probe: 5’-FAM- CCA CCT TCG GCT ACG GCC TG -TAMRA-3’; Beta globin: 5’- CCT ATC AGA AAG TGG TGG CTG G -3’ and 5’- TTG GAC AGC AAG AAA GTG AGC TT -3’, probe: 5’-FAM- TGG CTA ATG CCC TGG CCC ACA AGT A-TAMRA-3. Standards consisted of dilutions of DNA extracted from cell lines transduced with a single copy of the vector and DNA from control animal PBMC. Reactions were run using the ABI master mix
(Applied Biosystems, Branchburg, NJ) on the ABI Prism 7700 sequence detection system (Applied Biosystems) using the following thermal cycling conditions: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

**Results and Discussion**

*Reinfusion of preselected, GFP expressing CD34+ cells delays hematopoietic reconstitution.* Table 1 contains details regarding enrichment, cell dose, purity, vector assignment, and engraftment. Results illustrate that preselection and infusion of GFP expressing cells had a profound effect on the hematopoietic reconstitution and significantly delayed hematopoietic engraftment. The mean time to granulocyte engraftment in animals receiving preselected GFP-expressing cells was 28 days (standard deviation (SD) +/- 1 day, range, 27 -29 days) compared to 15.5 days (SD +/- 2.8 days, range, 12 – 20 days; p=0.01, Wilcoxon rank sum test) in 8 historic control animals. Consistent with data in other experimental models, the extended in vitro culture required for GFP expression prior to and the sorting manipulation itself may have prompted in vitro differentiation and compromised the repopulating ability.

*Preselection results in high short-term gene transfer.* Gene transfer was variable among animals, and we found no evidence that the overall gene transfer rate or the ability to select for transduced stem cells is affected by the presence or absence of SAR elements or the GFP variant. We determined overall marking at 4, 13, and 18 week follow–up time points by FACS to be: M00081 67/0.3/0.2%, J00116: 17/23/24%, and T00024 11/3.8/2.7%. Long-term gene transfer rates in two of these animals (not J00116) appeared to range below those previously attained without preselection. This is unlikely to be accounted for by silencing or immunologic clearance given the correlation of transgene expression (marking by FACS, Fig. 1 a) and proviral persistence (marking by real-time PCR, Fig1 b). The PCR/ FACS GFP marking ratios at given time points (1.9 – 2.8 on average per animal) were unaffected by fluorescence color (EGFP or EYFP) or the presence of SAR element within the vector. These ratios are within the limits of studies previously reported and consistent with large animal studies by Rosenzweig.
SAR elements enhance EGFP and EYFP fluorescence intensity in all hematopoietic lineages. The most striking finding in this study is the improvement in proviral expression measured as mean EGFP (EYFP) fluorescence (MFI) at serial time points after transplantation, Fig. 2a. Flow-cytometric analysis was performed separately in granulocytes, lymphocytes. The calculated ratio of MFI in granulocytes or lymphocytes for the SAR-containing over non SAR-containing arm at a given time-point (Fig. 2b) exceeds 1 in all three animals, indicating greater fluorescence intensity of the SAR than non SAR-containing vector regardless of EGFP (J00116), or EYFP (M00081 and T00024) fluorescence assignment. This difference in fluorescence intensity persists over time in the hematopoietic lineages analyzed demonstrating predominant SAR-mediated effects on the magnitude of transgene expression. The gain in SAR-mediated fluorescence intensity was also apparent in red blood cells (analyzed in 2 animals at fewer time-points, data not shown). Our observation that the SAR-mediated improvement in fluorescence intensity was relatively greater in T-lymphocytes than granulocytes is supported by reports from other investigators in vitro and in the murine xenograft model.

Findings of improved proviral expression from SAR-containing vectors are consistent with observations by Mielke and colleagues who located highly expressing proviral integrants in the genome in close proximity to SAR elements. Thus, the use of SAR elements may provide for improved stable expression from a single copy proviral integrant. It is noteworthy that alternate positioning of the SAR element as described by Hawley and colleagues may have additional safety benefits while maintaining improvements in expression.

In conclusion, we propose that SAR-mediated high-level transgene expression may provide an attractive strategy to improve long-term proviral expression, obviating the need for multi-copy integration and thus reducing the risk of mutagenic insertion. SAR elements should be considered for the design of improved stem cell gene therapy vectors.
Acknowledgements

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References


17. Lutzko C HS, Senadheera D, Carbonaro D, and Kohn DB. Addition of the B-interferon SAR leads to an orientation dependent increase in transgene expression in stem cells and their progeny. Mol Ther; 2002:s426


Table 1

<table>
<thead>
<tr>
<th></th>
<th>Enrichment purity (% CD34)</th>
<th>Fluorescence assignment/Purity (%)</th>
<th>Cells to animal/arm</th>
<th>ANC &gt;500 (day after transplant)</th>
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<tr>
<td>M00081</td>
<td>87</td>
<td>EGFP (98)</td>
<td>2.3 x 10^7</td>
<td>28</td>
</tr>
<tr>
<td>J00116</td>
<td>77</td>
<td>EYFP (89)</td>
<td>1.8 x 10^7</td>
<td>29</td>
</tr>
<tr>
<td>T00024</td>
<td>90</td>
<td>EGFP (97)</td>
<td>1.6 x 10^7</td>
<td>27</td>
</tr>
</tbody>
</table>

Transplantation and enrichment characteristics. Animals received 5 days of recombinant human (rh) stem cell factor (rhSCF, 50 µg/kg/day) and rh- granulocyte colony stimulating factor (rhG-CSF) 100 µg/kg/day subcutaneously (kindly provided by Graham Molineux, Amgen, Thousand Oaks, CA) before marrow harvest and myeloablative irradiation (TBI, 1,020 cGy). Animals received rhG-CSF, 100 µg/kg, intravenously, once daily from day 0 until their peripheral blood neutrophil counts were >1000/µl. Engraftment defines the first day of a neutrophil recovery to > 0.5 x 10^9/l in the peripheral blood. The table denotes the composition of autologous EGFP/ EYFP-marked graft products returned to animals M00081, T00024, and J00116 as noted. ANC; absolute neutrophil count, EGFP; enhanced green fluorescent protein, EYFP; enhanced yellow fluorescent protein.
Figure legends

**Fig. 1** Frequency of genetically modified cells in the peripheral blood after transplantation. Serial determinations of GFP marking (expressed as percent cells marked in the peripheral blood) were made by flow cytometry (1a), or polymerase chain reaction (PCR; = average copy number x 100 shown) (1b). Vector marking in cells transduced with SAR-containing vectors is shown in solid symbols, non SAR-containing vectors in open symbols. Animal designations and fluorescence assignment are noted: EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein.

**Fig. 2** Improved fluorescence intensity from SAR-containing vectors. 2a MFI in peripheral blood leukocytes (PBL) over time after transplantation. Fluorescence from cells transduced with SAR-containing vectors is shown in solid symbols, non SAR-containing vectors in open symbols. 2b To calculate ratios of MFI in SAR over non SAR-containing vector transduced cells we used mean fluorescence intensity (MFI) from forward and right-angle light scatter-gated granulocyte (circles) and lymphocyte (triangles) subsets using CELLQuest v3.1 as previously. A ratio of 1 implies equal fluorescence intensity between SAR vs non SAR vectors. A ratio of 10 denotes fluorescence 10 times brighter in cells transduced with the SAR-containing vector. Animal designations are noted.
Fig. 1

Flow-cytometry

$\text{GFP expression}$

$\begin{array}{c}
\text{SAR} \\
\text{Non-SAR}
\end{array}$

$\begin{array}{c}
\text{M00081} \\
\text{J00116} \\
\text{T00024}
\end{array}$

$\text{Days after transplantation}$

Real-time PCR

$\text{Proviral copy number (x 100)}$

$\begin{array}{c}
\text{SAR} \\
\text{Non-SAR}
\end{array}$

$\begin{array}{c}
\text{M00081} \\
\text{J00116} \\
\text{T00024}
\end{array}$

$\text{Days after transplantation}$
Fig. 2

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a. Days after transplantation

b. Days after transplantation
Scaffold attachment region-containing retrovirus vectors improve long-term proviral expression after transplantation of GFP-modified CD34+ baboon repopulating cells

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