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Transplantation of Murine Bone Marrow Stromal Cells Under the Kidney Capsule to Secrete Coagulation Factor VIII

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Ectopic cell transplantation has been studied as an alternative to whole organ transplantation or as a method to produce secretable proteins for genetic disorders. In this study, bone marrow stromal cells isolated from C57Bl/6 mice were genetically modified to express either lacZ- or B-domain-deleted human factor VIII. In vitro modification of the isolated bone marrow stromal cells was initially performed by transducing increased doses of VSV-G pseudotyped lentiviral vectors expressing lacZ. At a MOI of 25, all of the bone marrow stromal cells were X-gal positive, which maintained their ability to expand and differentiate prior to transplantation into mice. Extremely poor engraftment was observed in the liver, but transplantation of the bone marrow stromal cells expressing lacZ under the kidney capsule resulted in long-term viable X-gal-positive cells for at least 8 weeks (length of study). In vitro expression of human factor VIII was detected in a dose-dependent manner following bone marrow stromal cell with a factor VIII-expressing lentiviral vector. Transplantation of the factor VIII-expressing bone marrow stromal cells under the kidney capsule led to long-term therapeutic expression in the mouse plasma (1–3 ng/ml; $n = 4–5$ mice/group) for 8 weeks. This study demonstrated that ectopic transplantation of bone marrow stromal cells under the kidney capsule can be effective as a method to express secretable proteins in vivo.

Key words: Lentiviral vectors; Bone marrow stromal cells; Human factor VIII; Ectopic transplantation; Kidney capsule

INTRODUCTION

The development of cell-based therapies to treat organ failures, such as the liver, has been studied as an alternate method to whole organ transplantation (9,17, 21,28). Genetic modifications of primary hepatocytes have been performed by numerous investigators using a variety of different vector systems. One of the main problems is that these cells are difficult to transduce in culture and propagation of these cells cannot be maintained for longer than a few days (18,22). Moreover, these cells are not capable of efficient engraftment within the liver following vascular infusion. To augment the number of transplanted cells that remain viable for therapeutic efficacy, investigators have studied other extrahepatic sites for implantation, such as under the kidney capsule (20) or within the mesentery (31). However, the ectopic transplantation results in relatively short-term survival for primary hepatocytes.

To circumvent these issues, bone marrow-derived cells, which is a current area of intense research, may be used as an alternate source (6,29). Bone marrow stem cells can be divided into two main groups: hematopoietic and stromal cell lineages. In recent years, bone marrow stromal stem cells have been found to be readily cultured in vitro and expanded for prolonged periods (6,29). An additional factor that would benefit ex vivo gene manipulation in transplantable cells would be to design vector systems that would maximize long-term, therapeutic expression of a transgene of interest. Previous studies using naked DNA transfection of fibroblasts were performed by Roth et al. (31) prior to implantation into the upper omentum of mice. Although this approach was feasible, it is time consuming and required the need to acquire cells following antibiotic selection, which could compromise the cells to some degree. Towards this end, the use of viral vectors, specifically simple and complex retroviruses, would be ideal due to their intrin-

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sic nature of permanently integrating themselves into the host genome. VandenDriessche and colleagues used simple retroviruses based on murine Moloney leukemia virus to genetically modify human bone marrow stromal cells to produce coagulation factor VIII (3,4,34). However, recent studies have shown that complex retroviruses based on human immunodeficiency virus type 1 were significantly more effective in transducing most cell types compared to the MLV-based vectors (25), including human bone marrow mesenchymal stem cells (33). It remains to be determined whether genetically manipulated bone marrow stromal cells through the use of lentiviral vectors can maintain their survivability and capability to secrete proteins long term following transplantation.

The present study was designed to determine whether genetically modified bone marrow stromal cells using lentiviral vectors can be efficiently transplanted under the kidney capsule. These studies should allow us to better understand whether these cells will be useful as a factory to produce secretable proteins in vivo as a therapy.

MATERIALS AND METHODS

Animal Preparation

Male C57Bl/6 *scid* mice, 6–8 weeks old, were purchased from a commercial vendor (Jackson Laboratories). All animal protocols were performed according to the Louisiana State University animal facilities and NIH guidelines. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (2 mg/kg) prior to any surgical manipulation. Bone marrow stromal cells ($2–2.5 \times 10^6$ cells) were injected either directly into the liver or under the kidney capsule using a 1:1 mixture of RPMI and Matrigel. Mice were allowed to recover and had ad libitum access to food and water for the duration of the experiment.

Isolation of Primary Mouse Marrow Stromal Cells (MSCs)

Primary cultures of MSCs were obtained from the femurs and tibias of adult male C57Bl/6 mice as previously described (7,27). In brief, the long bones of mice were harvested aseptically and the ends of the bones were removed and placed into a tube. The tube was centrifuged, the bones were removed, and the cell pellet was resuspended prior to being triturated through a 21.5-gauge needle and filtered through a 70-μm nylon filter. This suspension was then plated and the adherent cells were allowed to settle for 24 h. Afterwards, the cells were washed with phosphate-buffered saline (PBS), and fresh medium was used to replenish the cells. Four weeks later, the cells were trypsinized, replated, and passed three additional times before characterization to obtain a pure population of murine MSCs.

Mouse Stromal Cell Transduction

Mouse stromal cells were plated in six-well dishes at a density of 5×10^4 cells/well. The cells were transduced in the presence of polybrene (8 μg/ml) with VSV-G pseudotyped lentiviral vectors expressing either lacZ- or B-domain-deleted human factor VIII. The medium was changed 24 h after infection, and the cells were allowed to incubate for an additional 24 h prior to the fixation with 0.1% glutaraldehyde. The cells were washed with PBS and then stained with 4% X-gal solution for 24 h. The cells were photographed to observe the dose at which the highest percentage of cells became X-gal positive without having gross morphological changes. The transductions were performed in three independent experiments.

Ex Vivo Transplantation of Bone Marrow Stromal Cells

LacZ and human factor VIII-expressing bone marrow stromal cells were trypsinized and resuspended in a 1:1 ratio of cold medium: liquid Matrigel (Becton Dickinson, Franklin Lakes, NJ). The cold medium contained RPMI with 0.7 mM EGTA and no calcium, which is the normal medium used to expand the bone marrow stromal cells. Because Matrigel polymerized quickly into a three-dimensional gel at room temperature, all the procedures involving Matrigel were performed at 4°C. Mice were anesthetized with a mixture of ketamine and xylazine, and then a flank incision was made in the mice. The kidneys were exposed and the cells were injected under the kidney capsule. The mice were sutured and allowed to recover for the duration of the experiment. At different time points, the kidneys were harvested and snap frozen on O.C.T. embedding medium on dry ice for sectioning. The sections were fixed in 0.1% glutaraldehyde, washed in PBS, and subsequently stained in 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; Calbiochem) solution overnight. The stained kidney sections were counterstained in hematoxylin and then dehydrated in ethanol, rehydrated in xylene, and cover slipped to look for X-gal-positive cells under the kidney capsule by light microscopy ($n = 3$/time point for animal sacrifice).

ELISA for Human Factor VIII

Mouse blood was obtained via retro-orbital bleeding and placed into sodium citrate buffer. Plasma was isolated following centrifugation, and the human factor VIII levels were measured by ELISA as described by the manufacturer's protocol (Affinity Biologicals, Hamilton, ON).

Lentiviral Vector Plasmids

The packaging construct, pCMVΔR8.74, and the envelope plasmid, pMD.G, have been previous described

(8). The transfer plasmid, pHRSVcEF1αhFVIIIR(+) W(+), containing the B-domain-deleted human factor VIII cDNA driven by the EF1α promoter, was cloned using standard techniques. pHRSVcPGKnlsLacZS1.4(+) W(+) containing nuclear localized lacZ driven by the PGK promoter was previously described (30). The rev-expressing plasmid, pRSV121, was provided by Dr. T. J. Hope (University of Illinois, Champaign, Il). The packaging plasmid, pCMV.gag.pol.RRE.bpA, was cloned by standard techniques.

Lentiviral Production and Assays

The VSV-G pseudotyped lentiviral vectors were generated as previously described in our lab (24–26,30). In brief, transient calcium phosphate transfection of 293T cells was performed using the following amounts of DNA: 10 μg transfer plasmid, 6.5 μg packaging plasmid, 5 μg rev-expressing plasmid, and 3.5 μg envelope plasmid. Chloroquine (25 μM) was added to the medium prior to transfection. Medium was replaced 12 h following transfection, and 36 h later the conditioned medium was harvested, filtered, and concentrated by ultracentrifugation. The concentrated viral pellet was resuspended in PBS without calcium and magnesium containing 10 μg/ml polybrene. The titer of the lentiviral vector stocks with the lacZ gene, serial dilution of concentrated virus was used to infect 5×10^5 HeLa in a six-well plate in the presence of polybrene (8 μg/ml). Blue cells were counted following X-gal staining to determine the lentiviral vector titer. For the lentiviral vector containing the factor VIII gene, the titer of the vector prep was determined by ELISA for the p24 Gag antigen concentration (Alliance; Dupont-NEN).

RESULTS

In Vitro Analysis on the Efficiency of Lentiviral Vector Transduction of Murine Bone Marrow Stromal Cells

Conditioned medium containing VSV-G pseudotyped lentiviral vectors was titered by end-point dilution on HeLa cells. In our preliminary experiment to assess the transduction efficiency of lentiviral vectors into the murine bone marrow stromal cells, we transduced a range of MOI from 1, 5, 10, 25, 50, and 100 in triplicate. We found highly efficient lentiviral vector transduction of murine bone marrow stromal cells isolated from C57Bl/6 mice was observed as determined by qualitative assessment of the cells following glutaraldehyde fixation and subsequent X-gal staining. Figure 1 demonstrates bone marrow stromal cells infected at a MOI of 25 with a lacZ-expressing lentiviral vectors. No X-gal-positive cells were detectable in vehicle or EGFP-expressing lentiviral vector transduced bone marrow stromal cells (data not shown). At the MOI of 25, no visible cytopathic effects were found following lentiviral vector transduction in the bone marrow stromal cells, whereas at higher MOI, the X-gal intensity was stronger, but less cells were found in the plate, suggesting cellular toxicity associated with the lentiviral vector likely due to the well-known fusogenic effects of the VSV-G envelope coat protein. One additional feature that we noted was that the transduced marrow stromal cells could maintain their plasticity in culture, because they could still differentiate into either adipocytes or osteocytes depending on the applied culture medium. We observed this phenomenon on three separate occasions using two different preparations of the lentiviral vector (data not shown).

Figure 1. In vitro transduction of murine bone marrow stromal cells with lentiviral vectors containing the bacterial lacZ gene. Bone marrow stromal cells (5×10^4 cells) were transduced with conditioned medium containing VSV-G pseudotyped lentiviral vectors (MOI 25). The lentiviral vector contained a nuclear localized lacZ gene driven by the phosphoglycerokinase promoter (PGK). The cells were fixed in glutaraldehyde and stained with X-gal solution.

Ex Vivo Analysis of Bone Marrow Stromal Cell Viability Injected Into the Liver

Initial experiments in our lab attempted to transplant the lentiviral vector-transduced bone marrow stromal cells into the parenchyma of the liver by either direct injection or by infusion through the portal vein. The marrow stromal cells (MOI 25) as shown in Figure 1 were expanded and were infused into mouse livers by portal vein injection using 1, 5, or 10×10^6 cells (n = 3–4 mouse/cell number). We found a rare number of X-gal-positive cells in the liver sections (data not shown), but we did observe a few X-gal-positive cells in the spleen. In our hands, the maximal number of cells that could be safely administered by direct portal vein infusion was 1×10^6 as higher numbers of cells appeared to result in mortality of the mice likely due to the blockage of blood vessels by the stromal cells, which are a relatively large cell in terms of diameter.

On the other hand, we found more abundant numbers of X-gal-positive cells compared to the portal vein approach, but the numbers were still extremely low when a direct injection of bone marrow stromal cells was performed into the mouse liver parenchyma. Figure 2A and B shows liver sections from two different mice (out of the four mice that were infused) demonstrating liver engraftment or cell fusion of the lacZ-expressing marrow stromal cells after 7 days following transplantation. In some liver sections, there was significant liver damage, likely due to the injection process either the needle or the placement of the cells into the liver parenchyma.

Ex Vivo Analysis of Bone Marrow Stromal Cell Viability Under the Kidney Capsule

Due to the low number of marrow stromal cells engrafted or fused in the liver, we examined an ectopic site to transplant the lentiviral vector-transduced marrow stromal cells. The space under the kidney capsule was an ideal storage place due to the large number of cells that can be readily implanted and its known survivability. Between 2 and 2.5×10^6 stromal cells mixed in a Matrigel solution were injected under each of the kidney capsules, and the kidneys were harvested every 2 weeks ($n = 3$/time point). The kidneys were sectioned and stained with X-gal solution to qualitatively determine the cell survivability following transplantation. Over the 8-week period, the transplanted cells appeared to survive as shown by the high number of X-gal-positive cells. Interestingly, ossicle formation was found to occur beginning at 2 weeks, and was clearly evident by week 4 throughout the subsequent time points. This type of differentiation has been previously shown to develop following hematopoietic stem cell transplantation under the renal capsule. No X-gal-positive cells were found in EGFP-transduced stromal cells, demonstrating the specificity of the lacZ expression (data not shown).

In Vitro Detection of Human Factor VIII in Lentiviral Vector Transduced Bone Marrow Stromal Cells

To determine whether our genetically modified bone marrow stromal cells could express human factor VIII in culture, we transduced and expanded these cells following transduction with an advanced third-generation lentiviral vectors expressing the B-domain-deleted human factor VIII cDNA driven by the EF1α promoter.

As shown in Figure 3, we found that human factor VIII could be dose-dependently produced following lentiviral vector transduction. Importantly, we found that these lentiviral vector-transduced marrow stromal cells could be frozen, thawed, and expanded at later time points (i.e., several weeks later) to produce similar levels of human factor VIII (hatched vs. dark gray bar). In addition, we found that additional transduction into the singly transduced MSCs produced higher levels of hFVIII. As a control cell line, we infected HeLa cells to demonstrate the ability of lentiviral vectors to transduce and express human factor VIII.

Overall, the results of our study demonstrated the potential benefit of genetically manipulating bone marrow stromal cells using VSV-G pseudotyped lentiviral vectors.

In Vivo Expression of Human Factor VIII in Mouse Plasma Following Transplantation Under the Kidney Capsule

Therapeutic levels of plasma human factor VIII was measured by ELISA in C57Bl/6 *scid* mice transplanted with 2.5×10^6 stromal cells/kidney ($n = 4$–5 mice/group) at 8 weeks postimplantation (Fig. 4). The bone marrow stromal cells were transduced either once or twice with factor VIII-expressing lentiviral vectors, and the double-transduced cells led to plasma factor VIII levels of 1.8 ± 0.4 ng/ml, which was ~50% higher than the single transduced cells at the 8-week time point. No detectable levels of plasma factor VIII was observed in vehicle or lacZ transduced bone marrow stromal cells under the kidney capsule. This may become a viable method to express coagulation factor VIII as well as other secretable proteins using this relatively simple ex vivo approach.

DISCUSSION

The present study demonstrates the relatively simple process of genetically modifying bone marrow stromal cells using advanced generation lentiviral vectors pseudotyped with the VSV-G envelope coat protein. To transplant cells following genetic manipulation, it is imperative to obtain cells that have stable integration of the transgene of interest, otherwise any cellular proliferation by the implanted cells will result in long-term loss of signal. Previous studies have used transfection of naked DNA into isolated primary fibroblasts to express secretable proteins, such as human factor VIII, following implantation into the mesentery (31). However, these isolated cells needed to be selected by antibiotic resistance over several week period prior to transplantation. This type of approach limits the types of cells that can be manipulated due to the long time course needed to isolate a pure population of modified cells.

Towards this end, viral vectors that have intrinsic capabilities to integrate into the host genome are preferred, but have not been widely tested on cells, such as bone marrow stromal cells. Simple and complex retroviral vectors appear to be highly useful for these types of experiments because cell division would not adversely affect the ability of this vector system to persist, unlike other episomal vector systems. Previous studies have used murine Moloney-based retroviral (MLV) vectors to

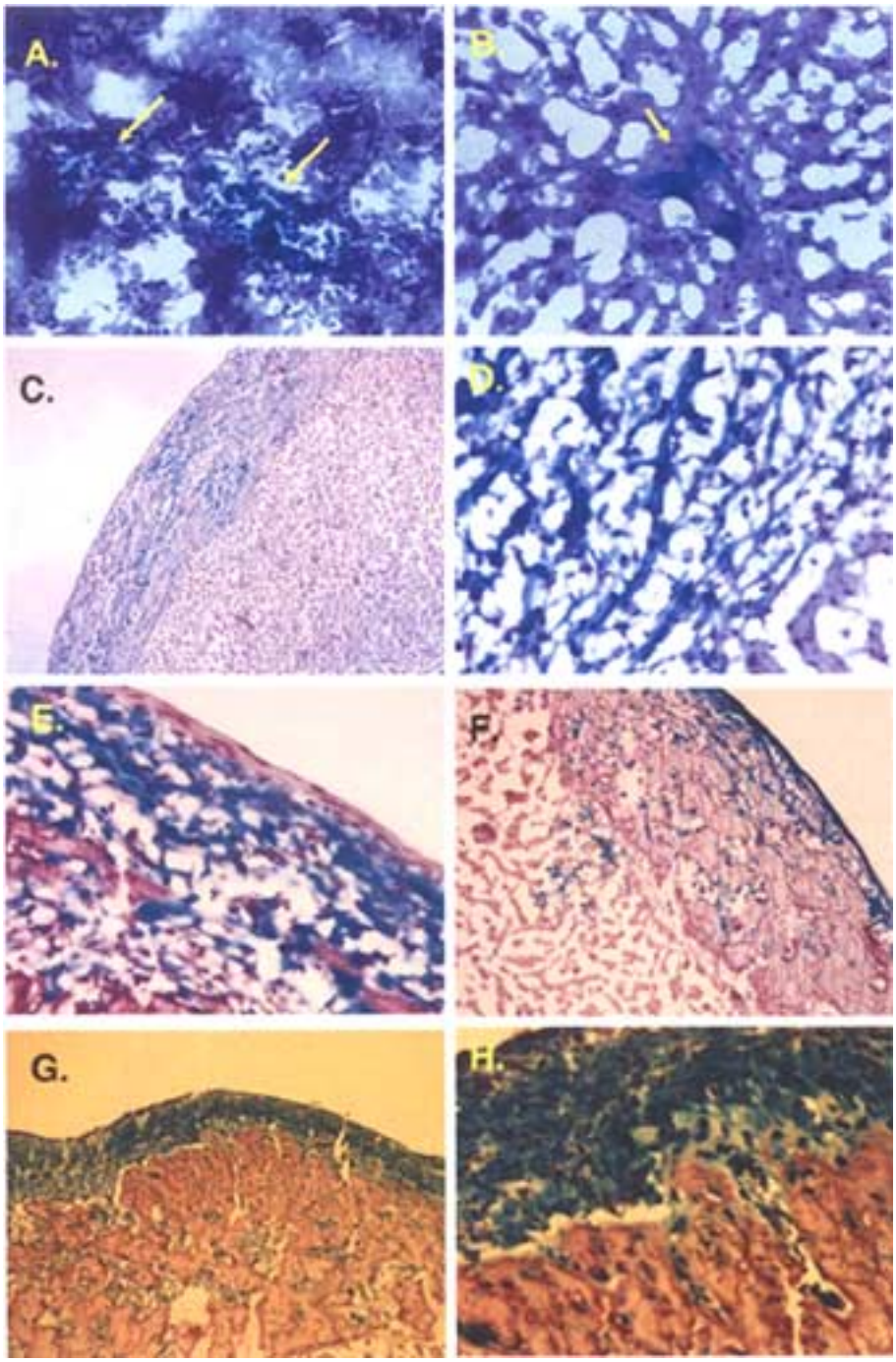

Figure 2. In vivo expression of lacZ following transplantation of bone marrow stromal cells transduced with VSV-G pseudotyped lentiviral vectors into the liver parenchyma and under the kidney capsule. Mouse livers were harvested 7 days following the direct injection of the bone marrow stromal cells (1×10^6 cells) into the liver parenchyma. Liver sections (8 μm) were stained with X-gal to examine the engraftment of the murine bone marrow stromal cells in C57Bl/6 mice as shown in (A) and (B) (magnification 400×). (C–I) The X-gal-positive bone marrow stromal cells at different time points after transplantation under the kidney capsule. Kidney sections (8 μm) were stained with X-gal and imaged at week 2 (C, 10×) and (D, 400×), week 4 (E, 400×), week 6 (F, 100×), and week 8 (G, 10×) and (H, 400×) following the transplantation of the bone marrow stromal cells ($n = 3$–4 mouse livers and kidneys/time point). Arrows in (A) and (B) point to the X-gal-positive cells found in the liver parenchyma following direct injection into the liver.

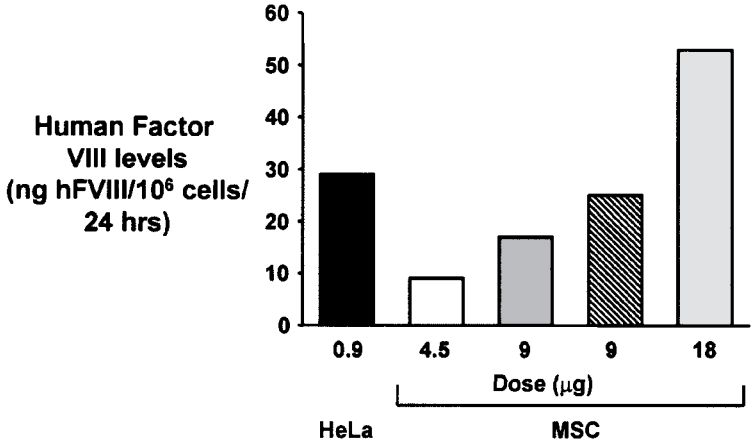

Figure 3. In vitro transduction of murine bone marrow stromal cells with lentiviral vectors containing the human factor VIII (B-domain deleted) gene. Bone marrow stromal cells (5×10^4 cells) were transduced with increasing doses (between 4.5 and 18 μg p24 Gag protein) of VSV-G pseudotyped lentiviral vectors containing the B-domain-deleted human factor VIII gene. Medium was collected 96 h following transduction into bone marrow stromal cells and the medium was tested for the detection of human factor VIII levels by ELISA. HeLa cells (black bar; 0.9 μg p24) were used as a control for lentiviral vector transduction and human factor VIII expression. The hatched bar shows that MSCs are readily frozen and expandable allowing for repeated use after infection (in comparison to gray bar).

transfer expressible transgenes into bone marrow stromal cells (3,4,12,32,34). Schwarz et al. (32) demonstrated that MLV vectors can effectively transduce rat marrow stromal cells to produce two different genes that were necessary to efficiently synthesize L-DOPA. These stromal cells were capable of expansion and proliferation without any loss of its ability to produce the gene of interest. In other studies, MLV vector-transduced bone marrow stromal cells were found to readily secrete significant levels of human factor VIII in vitro (3,4,34). However, in vivo studies by Chuah et al. (4) found that retroviral vector-transduced stromal cells expressed factor VIII transiently following splenic implantation. One reason for this is the well-known transcriptional silencing that occurs through a phenomenon known as position effect variegation (23), which precludes using these

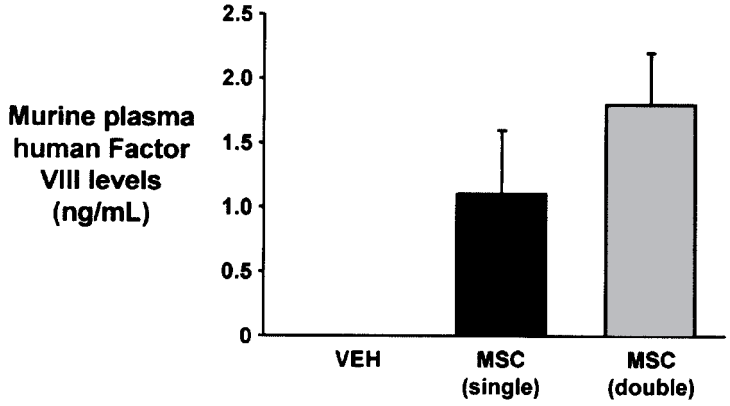

Figure 4. Human factor VIII expression in mouse plasma following transplantation of bone marrow stromal cells under the kidney capsule. Mouse plasma was isolated by retro-orbital bleeding of the C57Bl/6 *scid* mice transplanted with the human factor VIII-expressing bone marrow stromal cells (MSC; $2.2–2.5 \times 10^6$ cells/kidney capsule). The mouse plasma was harvested 8 weeks after the implantation of the MSCs. The MSCs were treated with either vehicle (VEH) or transduced once (MSC single) or twice (MSC double) with VSV-G pseudotyped lentiviral vectors ($n = 4–5$ mice/group).

vectors for long-term expression studies. Although early generation complex retroviruses have shown downregulation problems, it has been shown that deletions within the U3 region can alleviate the promoter interference (10). A more recent issue that may preclude the use of simple retroviruses is the leukemogenesis that developed in the several of the young children enrolled in the X-linked SCID trials following genetic manipulation of human hematopoietic stem cells using MLV-based retroviral vectors (13).

Lentiviral vectors, which are complex retroviruses, have been found to be more efficient than MLV-based retroviral vectors in transducing a wider number of cell types provided that the VSV-G envelope coat protein is utilized during vector production (25). The findings in our study demonstrate that VSV-G pseudotyped lentiviral vectors are a reliable and effective vehicle to transduce isolated bone marrow stromal cells from mice, and this suggests that this method may have potential as a cell therapy application in humans. In comparison, a previous study by Totsugawa et al. (33) found that an early generation lentiviral vector system could transduce bone marrow mesenchymal stem cells (~40% efficiency) originating from humans.

Although there are safety questions on the use of lentiviral vectors in humans, there is recent in vitro promoter trapping experiments that have shown an integration pattern by lentiviral vectors that differs greatly from MLV-based vectors, in which lentiviral vectors were found to integrate distal to the promoter (5). This type of integration pattern should minimize the possibility of insertional mutagenesis leading to the activation of oncogenes. The other important point to make is that lentiviral vectors are now in Phase I/II of human clinical trials in which the investigators are attempting to address the issue of safety and efficacy of this system as a future medical therapeutic (1,14,15). As far as we are aware, our current study is the first to use modified lentiviral vectors to transduce bone marrow stromal cells to express a secretable protein, such as human factor VIII, in vivo by transplanting the cells under the kidney capsule.

Although the preferred site of coagulation factor VIII production would be hepatocytes, which are the normal synthesis site in the body, the ability of hepatocytes or other types of cells, including stems cells, has been extremely difficult to achieve. However, there is a recent study demonstrating that cells committed to the myelomonocytic lineage can be found to fuse to hepatocytes following splenic transplantation (36). The isolation of cells for this method is laborious and time consuming, in contrast to the techniques described in this current study using easily obtainable bone marrow stromal cells. In our approach, the transplantation of marrow stromal cells under the kidney capsule has been shown to allow engraftment of primary cells for a wide range of study from basic biological processes involved in differentiation to its use as a protein secretion factory (11,20,35). Previous studies by investigators have shown that bone marrow cells can be effectively transplanted beneath the kidney capsule for over 6 months (i.e., up to 192 days) (35). Moreover, Gurevitch et al. (11) have suggested that the stromal cell microenvironment helps to promote engraftment in ectopic sites, such as under the kidney capsule. Other investigators have transplanted hepatocytes into the kidney capsule to produce secretable proteins (20). As an alternative ectopic site for the transplantation of bone marrow stromal cells in the production of human factor VIII could be into the subcutaneous fat. A recent study by Ogata et al. (19) using a lentiviral vector derived from monkeys found that human factor VIII can be secreted from adipocytes. Because the lentiviral vector-transduced bone marrow stromal cells can still differentiate into adipocytes, the transplantation into fat pads may be another alternative method to produce secretable proteins, especially because the injection sites would be more prevalent than the kidney capsule.

Our present study demonstrates that bone marrow stromal cells are flexible in the use as a factory to express secretable proteins compared to other cells, such as primary hepatocytes. First, the transduction efficiency into mouse bone marrow stromal cells is significantly higher than murine primary hepatocytes (18,22). Second, bone marrow stromal cells can be serially passaged as well as frozen prior to expansion in cell culture for transplantation into an ectopic site. Third, genetic manipulation with lentiviral vectors appears to be readily efficient compared to primary hepatocytes, which can have significant toxicity associated with the vector preparations even at subsaturating doses.

Future studies will need to consider manipulations of the coding sequence to maximize circulating levels of a secreted protein (i.e., insertion of a proteolytic site at amino acid 1648) (2). Recent studies by Miao et al. (16) have described a modified B-domain-deleted human factor VIII that can be secreted 15–25 times more efficiently than the full-length human factor VIII protein in vitro and in vivo. The optimization of a vector system to genetically modify a cell type, such as bone marrow cells, as well as inserting a highly efficient protein will be important to determine the utility of cell-based transplantation experiments.

In all, we have shown that bone marrow stromal cells isolated from C57Bl/6 mice can be effectively transplanted under the kidney capsule for at least 8 weeks and survive in sufficient numbers to produce low but therapeutic levels of secretable coagulation factors into the circulation. Future studies will need to optimize and enhance the secreted protein levels, and potentially mod-

ify the cells in such a manner to differentiate into a cell type that may be more conducive for factor VIII production.

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