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Single-chain antibody-mediated gene delivery into ErbB2-positive human breast cancer cells

Xuguang Li,[1] Patricia Stuckert,[1] Irene Bosch,[1] James D. Marks,[2] and Wayne A. Marasco[1,3]

[1]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts 02115; [2]Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, California 94110; and [3]Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115.

Targeted gene transfer by nonviral vectors can be achieved through incorporation of specific ligand(s) into the vectors. In this study, the effects of incorporation of an anti-ErbB2 single-chain antibody fragment (ScFv) into nonviral vectors for targeted gene delivery were investigated. The ML39 ScFv, selected from a human ScFv phage display library and affinity matured *in vitro* ($K_d = 1 \times 10^{-9}$ M), was used as ligand specific for the extracellular domain of the tumor surface protein, ErbB2. Two approaches were taken: (a) development of a vector that is composed of a bifunctional fusion protein capable of binding DNA with the ErbB2-specific ML39 ScFv at its N-terminus and a truncated form of human protamine at its C-terminus, and (b) formulation and evaluation of delivery vectors consisting of three independent components including ML39 ScFv, protamine, and cationic lipids. We demonstrate that fusion proteins comprised of the ML39 ScFv and a truncated form of protamine, denoted as ScFv-P-S, can selectively deliver exogenous DNA into ErbB2(+) cells, with an 8- to 10-fold increase in expression levels of the luciferase reporter gene in ErbB2(+) cells as compared to ErbB2(−) cells. In addition, vectors formulated by appropriately mixing DNA, ScFv, protamine, and lipids *in vitro* could even more efficiently deliver the reporter gene into ErbB2(+) cells with approximately 5-fold increase in gene expression in ErbB2(+) cell as compared to ErbB2(−) cells. Expression and refolding of the ScFv fusion proteins, in addition to determination of optimal conditions for vector development using these approaches, are discussed.
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Targeted gene therapy requires that the therapeutic gene(s) be expressed in a cell- and/or tissue-specific manner. Although viral vectors can efficiently deliver exogenous gene into a relatively defined cell population, further alterations toward improving target specificity often result in reduced infectivity of the recombinant virus.[1] Other potential problems associated with viral vectors include immunogenicity, risk of insertional mutagenesis, difficulty in large-scale production, and size restrictions for exogenous DNA.[2,3] These limitations of viral vector systems substantially restrict their clinical application. In recent years, nonviral vectors, such as cationic liposome and polymer complexes,[4,5] have received increasing attention because of advantages such as lack of immunogenicity, ease of preparation, and relative safety.[4,5] Furthermore, targeted delivery of foreign genes into a defined cell population can also be achieved if a receptor-binding ligand is incorporated into the synthetic vector.[6–14] It is believed that these DNA/vector complexes are taken up by the cell through receptor-mediated endocytosis and dissociated in the lysosome, resulting in release of exogenous DNA into the cytoplasm prior to translocation and expression of the transferred DNA in the nucleus of the cells.[8,10,13]

Various cell surface markers and/or receptors, including those associated with cancer,[6–14] have been explored as potential targets for nonviral gene delivery. One of the tumor-associated receptors studied over the past years is ErbB2,[13,14] which is a member of the EGFR family.[15] Genetic studies reveal that ErbB2 is frequently amplified and/or overexpressed in a variety of human cancers.[15–18] Its overexpression has also been correlated with poor prognosis and reduced survival of patients with breast and ovarian cancers.[15–18] In addition, biochemical studies indicate that ErbB2 possesses an extracellular domain capable of internalizing ligand.[17] This property has been explored in the context of targeted gene transfer into breast cancer cells by integrating ErbB2-specific single-chain antibody fragment (ScFv) into the delivery vector as a portion of DNA-condensing fusion protein.[13,19] Whereas specific gene transfer into ErbB2(+) breast cancer cells has been achieved by this approach, murine ErbB2-specific ScFv (the specific ligand), in addition to yeast Gal4 (the DNA-condensing module), was employed in these studies. Such vectors can be immunogenic in the human host, thereby compromising their potential therapeutic value.

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Address correspondence and reprint requests to Wayne A. Marasco, MD, PhD, Dana-Farber Cancer Institute, Department of Cancer Immunology and AIDS, 44 Binney Street, JFB 824, Boston, MA 02115. E-mail address: wayne_marasco@dfci.harvard.edu

We previously reported the development of a bifunctional fusion protein with a human Fab (specific for HIV-1 envelope protein) at the N-terminus and a DNA-condensing polypeptide human protamine at the C-terminus.[14] Fusion proteins expressed and purified from COS cells were able to condense plasmid DNA and selectively deliver them into HIV-1–infected cells. However, difficulty in preparing these fusion proteins from COS cells hampered their practical application. In this report, we extended this study by using two approaches: (a) development of a bifunctional fusion protein with the human ML39 ScFv specific for ErbB2 at its N-terminus and a truncated form of human protamine at its C-terminus that has specific targeting and DNA-condensing capabilities and, (b) formulation of delivery vectors consisting of three individual components including protamine, ML39 ScFv, and cationic lipids. We demonstrate that fusion proteins comprised of the human ML39 ScFv and a truncated form of human protamine, denoted ScFv-P-S, can selectively deliver exogenous DNA into ErbB2(+) cells, with a 8- to 10-fold increase in selected gene expression levels in ErbB2(+) cells as compared to that in ErbB2(−) cells. We also report here that vectors formulated by appropriately mixing DNA (D), protamine (P), ML39 ScFv (S), and lipids (L), designated DNA/protamine/ScFv/lipid (DPSL), can more efficiently deliver exogenous DNA into ErbB2(+) cells (compared to ScFv-P-S) and with substantial selectivity, i.e., 5-fold increase in gene expression in ErbB2(+) cells compared to ErbB2(−) cells.

METHODS

Cloning, expression, purification, and characterization of ScFv and ScFv–protamine proteins

The anti-ErbB2 ML39 ScFv gene sequence was removed from pSyn-1 ML39[20] and cloned into pAcGP67B baculovirus transfer vector at restriction sites *Nco*I and *Not*I to allow secretion of the ScFv and ScFv fusion proteins into the medium. A six-His tail was also engineered at the C-terminus of the peptide to facilitate purification. The full-length human protamine sequence has been described previously.[14] The sequence for the truncated form of human protamine comprising amino acids 8 through 29 is as follows: RSQSRSRYYRQRQRSRRRRRRS. The sequence was placed downstream of the ML39 ScFv, with a His×6 tail at the C-terminus. Recombinant baculovirus was generated by using the BaculoGold system from Pharmingen (San Diego, CA).

To isolate recombinant proteins from culture supernatants, supernatants from SF21 cell cultures infected with the recombinant viruses were collected by centrifugation at 2000 rpm for 20 minutes at 4°C. The proteins were precipitated by addition of equal volume of saturated ammonium sulfate to the harvested culture supernatant, followed by incubation of the mixture at 4°C overnight. The proteins were then pelleted by centrifugation at 10,000×*g* for 30 minutes at 4°C. The resulting pellets were resuspended in phosphate-buffered saline (PBS)/10% glycerol, followed by extensive dialysis with 5% glycerol/PBS. The dialyzed protein preparations were then applied to a Probound nickel-chelating column (Invitrogen, San Diego, CA). Recombinant ScFv proteins were purified under native condition using increasing concentrations of imidazole in the elution buffer. The collected protein fractions were then analyzed for purity using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. The purified proteins were dialyzed against PBS plus 5% glycerol to remove imidazole.

To isolate protein from cellular extracts, cells were dissolved in 6 M guanidine hydrochloride (GuaHCl) for 30 minutes on ice, followed by brief ultrasonication. Insoluble cellular debris was then removed by centrifugation at 12,000×*g* for 30 minutes at 4°C. The resulting protein preparations were then applied to a Probound column (Invitrogen, Carlsbad, CA) under denaturing conditions (6 M GuaHCl). Recombinant proteins were then eluted by increasing concentrations of imidazole in the presence of 6 M GuaHCl. The eluted proteins were refolded using methods previously described[21–24] with slight modifications. Briefly, the samples were first dialyzed against Buffer I, which consists of PBS containing linear decreasing concentrations of GuaHCl (from 6 to 0 M) generated by a gradient maker (Fisher Scientific, Atlanta, GA) over a period of 48–72 hours, followed by dialysis against Buffer II, which consists of 5% glycerol, 0.5 M arginine, 1 mM EGTA, and 1 mM glutathione in reduced and oxidized form at 4°C for 48 hours. Afterwards, the buffer was changed to PBS/5% glycerol by dialysis. Finally, the protein preparations were concentrated and stored at 4°C with 0.005% sodium azide, or aliquoted and frozen at −70°C.

Formulation of DPSL and transfection of cells

Formulation was generally performed as follows, unless specified: in 75 μL of serum-free DMEM and 1 μg of DNA were preincubated with 0.1 μg of salmon protamine sulfate, grade II (Sigma, St Louis, MO) and 4 μL of ScFv (1 μg/μL) at room temperature for 15 minutes, followed by addition of 20 μL of Dotap (Boehringer Mannheim, Indianapolis, IN) prior to another 15-minute incubation. The complex was added drop by drop into the cells, followed by incubation at 37°C for 4 hours. The cells were then washed twice with PBS prior to incubation for 48 hours with fresh DMEM plus 10% fetal calf serum (FCS) at 37°C. The amount and type of each reagent used in vector development were determined based on resistance to DNase I treatment and levels of transgene expression (see *Results*).

DNAse protection assay

Protection of DNA by protein and/or lipid was performed by treating the complex with excessive amount of DNase (40–80 U of DNase I per microgram of DNA). After incubation at 37°C for 2 hours, the reaction mixture was then added with SDS to final concentration of 1% and Proteinase K at 1 mg/mL, followed by incubation at 37°C for 2 hours. The mixture was then twice extracted with phenol/chloroform for isolation of DNA fragments. The aqueous phase containing the DNA fragments following extraction was then analyzed

by ethidium bromide staining of 1% agarose gel and photographed under UV light.

Flow cytometry

To determine binding of ScFv to the ErbB2 molecules, we used ErbB2(+) cells (SKBR3) and ErbB2(−) cells (MCF7) in a flow cytometry assay. Briefly, the cells were first detached from the bottom of tissue culture dishes by using a mild method for protection of the cell surface proteins, i.e., incubation of the cells in enzyme-free cell dissociation buffer (Life Technologies, Rockville, MD) for 20 minutes, followed by washing with PBS containing 5% FCS. The cell suspension was then incubated with ScFv (2 μg/mL for 10^6 cells) for 30 minutes on ice. The cells were then washed 3× with PBS/5% FCS, followed by further incubation with murine anti-His tag monoclonal antibody (Babco, Berkeley, CA). Finally, the cells were washed 3× with PBS/5% FCS prior to a 30-minute incubation with FITC-conjugated goat antimouse IgG. The reaction was terminated by three additional washes with PBS/5% FCS and fixed in 2% formaldehyde/PBS. Positive cells were scored by FACS analysis.

Analysis of transgene expression

The SKBR-3 and MCF7 cell lines were maintained at exponential phase before transfection with DNA/vector complexes was performed. After addition of the complex, the cells were incubated for an additional 48 hours before being harvested for luciferase analysis using the detection system from Promega (Madison, WI). In brief, cells were washed twice with PBS before being lysed in buffers supplied by the manufacturer. Protein concentration was determined using Bradford assay kit (Bio-Rad, Hercules, CA). Equal amount of proteins from each sample were used for luciferase analysis according to procedures recommended by the manufacturer. To confirm the specificity of ScFv-mediated gene transfer, monoclonal antibody against ErbB2 (Santa Cruz Biotechnology, Santa Cruz, CA), or in some cases ScFv,[20] was preincubated with the cells for 10 minutes to block the ErbB2 receptor before transfection with DNA/vector complex was initiated.

RESULTS

Expression, purification, and binding activity of anti-ErbB2 ScFv and ScFv–protamine fusion proteins

Recombinant baculovirus expressing either ML39 ScFv or ML39 ScFv–protamine fusion proteins was used to infect SF-9 cells. The pAcGP67B transfer vector (Pharmingen) harbors the gp67 signal peptide that mediates forced secretion of recombinant proteins. We collected supernatants from the baculovirus cultures and performed Western blot on these samples. Figure 1A shows that abundant ScFv was detected in the supernatant; however, the ScFv fusion proteins, regardless of whether it is the full-length form (ScFv-P-L) or the truncated form (ScFv-P-S), were not detected in the supernatant (Fig 1A). Cell lysis in SDS-PAGE sample buffer followed by Western blot analysis of

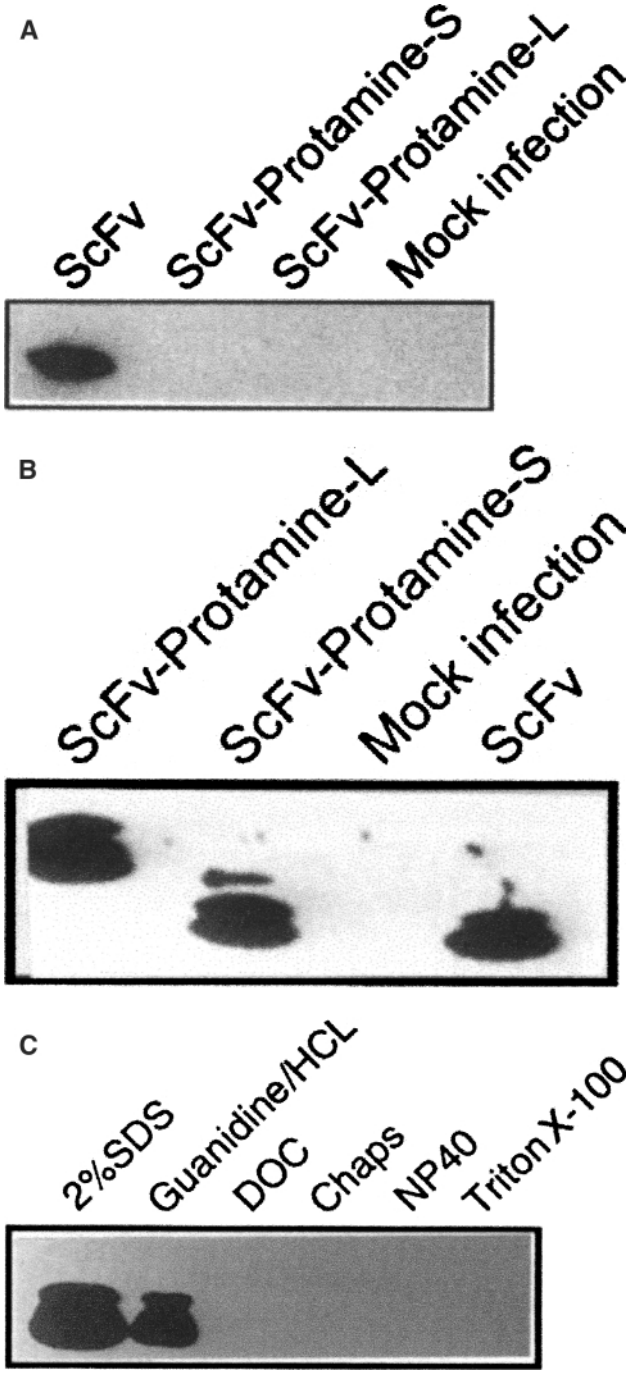


Figure 1. Detection of ScFv and ScFv–protamine fusion proteins. Expression of recombinant protein (ScFv) and fusion proteins, including ScFv-P-S (the truncated fusion protein) and ScFv-P-L (the full-length fusion protein), was determined in Western blot assays. Protein samples were fractionated in SDS-PAGE followed by Western blot analysis using MAb against His×6 tag (Babco). **Panel A:** Detection of recombinant proteins in supernatants of baculovirus culture. **Panel B:** Detection of recombinant proteins in cellular extracts. **Panel C:** Detection of recombinant protein ScFv-P-S in cellular extracts from cells lysed with various reagents including 2% SDS, 6 M GuaHCl, 0.5% deoxycholate, 0.5% chaps, 1% NP40, or 1% Triton X-100.

the lysates revealed that the fusion proteins were trapped intracellularly (Fig 1B). In addition, only strong denaturants such as 2% SDS and 6 M GuaHCl were able to extract the fusion proteins ScFv-P-S (Fig 1C). As 2% SDS resulted in irreversible denaturation of the proteins (data not shown), we chose 6 M GuaHCl to extract fusion proteins from the cell pellets, whereas the ScFv was purified from supernatants under native conditions. As is shown Figure 2A, the ScFv was purified by elution with 25–100 mM imidazole to near homogeneity under native conditions where PBS was used as buffer. Purification of the truncated form (ScFv-P-S) and the full-length fusion protein (ScFv-P-L) was performed in the presence of 6 M GuaHCl. Figure 2B shows that the ScFv-P-S was purified by elution with 50 mM imidazole to near homogeneity. Figure 2C shows that the ScFv-P-L was also purified to near homogeneity, albeit at a much lower yield as demonstrated in the gel stained with Coomassie blue (Fig 2C).

As the ScFv fusion proteins were purified under denaturing condition (6 M GuaHCl), the protein samples were dialyzed against PBS over a period of 48 hours at 4°C in an attempt to renature the proteins. The proteins harvested from dialysis were then assayed for their binding capabilities to ErbB2 in FACS analysis. As shown in Figure 3, neither ScFv-P-S (panel A) nor ScFv-P-L (panel B) had any binding activities for ErbB2(+) SKBR3 cells, whereas ScFv purified under native conditions displayed strong binding capability to SKBR3 cells (panel C). Panel D is a negative control in which no ScFv was added. These results indicate that fusion proteins were misfolded. Thus, alternative protocols were used for renaturation of these fusion proteins (see below).

The ScFv fusion proteins were first dialyzed with refolding buffer I containing linearly decreasing concentrations of GuaHCl (from 6 to 0 M) over a period of 48 hours at 4°C, followed by extensive dialysis against refolding buffer II, which consists of 5% glycerol, 0.5 M arginine, 1 mM EGTA, and 1 mM glutathione in reduced and oxidized form at 4°C for additional 48 hours (see *Materials and Methods* for details). Finally, the buffer was changed to PBS/5% glycerol by dialysis. Figure 4A shows results from FACS analysis on the "refolded" proteins for their capability to bind to ErbB2(+) SKBR3 cells. Panel A represents results from ScFv-P-S binding before the refolding treatment; panel B depicts ScFv-P-S collected from dialysis against refolding buffer I, showing that ScFv-P-S has partially regained its binding capability to ErbB2. Clearly, it was only after the final step of refolding (dialysis against refolding buffer II) that ScFv-P-S has fully regained its binding capability (panel C). However, refolding of the full-length fusion protein (ScFv-P-L) was problematic because the two-step refolding process (panel E for refolding with buffer I and panel F for buffer II) only partially restored binding capability of ScFv-P-L when compared with ScFv-P-L before refolding treatment (panel D). Also shown in Figure 4A is the positive control in which monoclonal antibody was used in place of ScFv fusion proteins (panel G), and the negative control in which only FITC-conjugated goat antimouse IgG was used (panel H). These above results indicate that ML39 ScFv can be easily isolated and purified

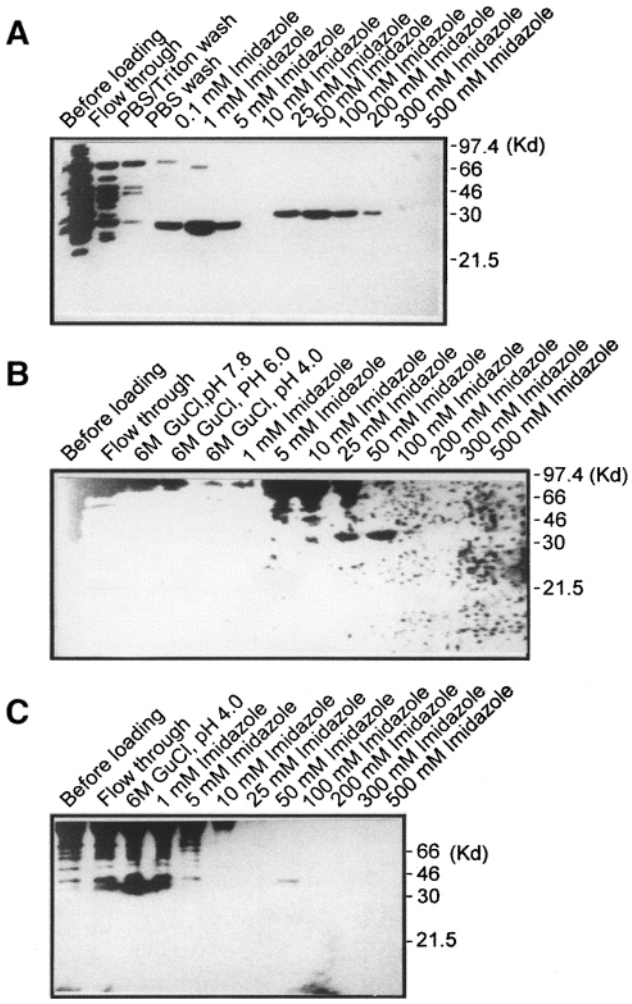


Figure 2. Detection of recombinant proteins in fractions of the purification process. Fractions of proteins collected during the purification process were loaded onto 12% SDS-PAGE followed by staining with Coomassie blue. **Panel A:** ScFv secreted into the culture supernatants was purified under native condition; ScFv was eluted with imidazole at concentrations ranging from 25 to 200 mM. **Panel B:** ScFv-P-S (the truncated form of fusion protein) was purified in the presence of 6 M GuaHCl; ScFv-P-S was optimally eluted with imidazole at 25–50 mM. **Panel C:** ScFv-P-L (the full-length fusion protein) was purified in the presence of 6 M GuaHCl; ScFv-P-L was best eluted with imidazole at 50 mM.

in large quantities (1–2 mg/L of baculovirus culture) and retains good binding capabilities to ErbB2(+) SKBR3 cells. However, fusion proteins ScFv-P-S and ScFv-P-L were both trapped intracellularly. These ScFv fusion proteins could only be extracted from cell pellets with 6 M GuaHCl. The yield for the fusion proteins was low with ScFv-P-S and ScFv-P-L at 20 and 5 μg/L, respectively. In addition, the ScFv fusion proteins need a lengthy refolding process for the binding activity of the truncated form, ScFv-P-S, to be fully recovered, whereas the binding activity of the full length fusion protein, ScFv-P-L, was only partially recovered.

The abilities of ScFv-P-S and ScFv-P-L to bind DNA were also tested. To this end, 1 μg of DNA was mixed

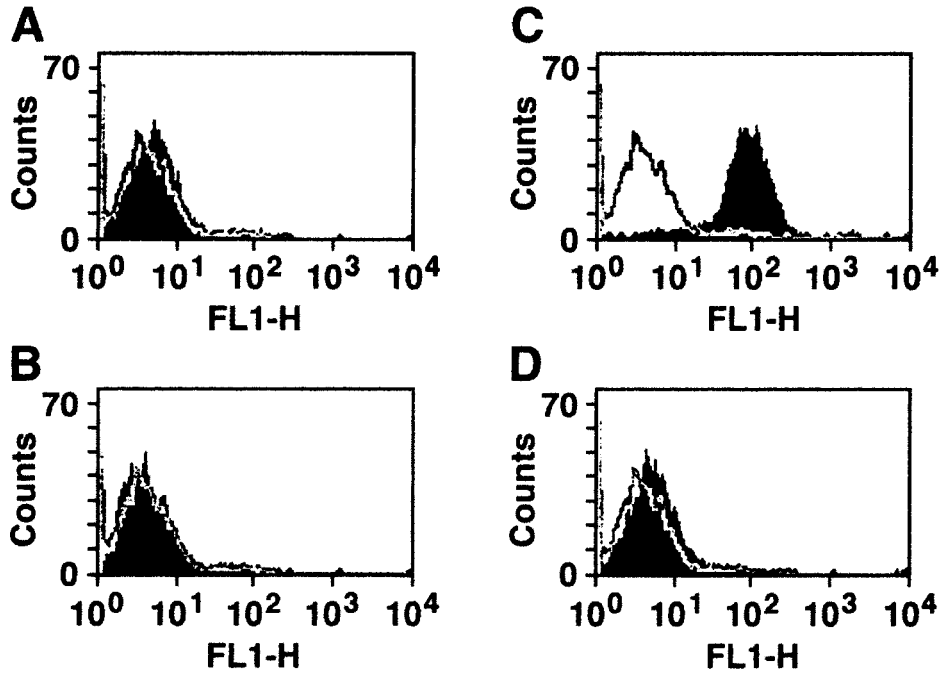


Figure 3. Determination of binding capability of recombinant proteins in FACS assays. The ability of fusion proteins to bind to ErbB2(+) SKBR3 cells was detected by FACS analysis. Purified recombinant proteins (2 μg/mL per 10^6 cells) were incubated with ErbB2 expressing SKBR3 cells, followed by additional incubation with MAb against His×6 tag prior to final incubation with FITC-conjugated goat antimouse IgG (Sigma). **Panel A:** ScFv-P-S binding. **Panel B:** ScFv-P-L binding. **Panel C:** ScFv binding. **Panel D:** Negative control in which no recombinant protein was added.

with various amounts of ScFv-P-S or ScFv-P-L and incubated at room temperature for 15 minutes. The reaction mixture was then analyzed on 1% agarose gel. As is shown in Figure 5, both ScFv-P-S and ScFv-P-L were able to shift DNA in a concentration-dependent manner, whereas ScFv itself displayed no DNA binding capability. Presumably, ScFv-P-L is better able to bind DNA than ScFv-P-S, most likely because the former contains many more positively charged amino acids (24 arginines for the full-length human protamine and 12 arginines for the truncated protamine polypeptide).[14] Taken together, these results demonstrate that the fusion proteins, ScFv-P-S and ScFv-P-L, are bifunctional, i.e., their ScFv portion can recognize ErbB2, and the protamine portion can bind to DNA.

ScFv fusion proteins can selectively deliver DNA to ErbB2(+) cells

We previously reported that a fusion protein comprised of a recombinant Fab fragment of human anti–HIV-1 gp120 genetically linked to human protamine can selectively deliver an intracellular toxin gene into HIV-1–infected cells.[14] Therefore, we next investigated whether ScFv-P-S and ScFv-P-L could deliver exogenous DNA (pcDNA-Luc, a luciferase expression vector) into ErbB2(+) SKBR3 cells. We held the amount of pcDNA-Luc constant at 1 μg, to which various amounts of ScFv-P-S or ScFv-P-L were added and incubated at room temperature for 30 minutes. The mixture was then added to ErbB2(+) SKBR-3 cultures and incubated for an additional 4 hours. The cells were subsequently washed with PBS, followed by addition of fresh media, and cultured at 37°C for 48 hours, at which time the cells were harvested for luciferase analysis.

As is shown Figure 6, panel A, both ScFv-P-S and ScFv-P-L were able to deliver pcDNA-Luc into the ErbB2(+) SKBR3 cells at a concentration range between 1 and 10 μg when mixed with 1 μg of pcDNA-Luc. At higher ratio between protein and pcDNA-Luc, expression of transgene decreases as excess, free fusion proteins block the ErbB2 receptor and inhibit uptake of the DNA/protein complex by the cells (see below). Although it may be expected that the full-length fusion protein ScFv-P-L is more efficient in delivering exogenous DNA as compared to the short-form fusion protein ScFv-P-S based on its increased ability to condense DNA (Fig 5), the results in Figure 6, panel A show that ScFv-P-L is less efficient than ScFv-P-S in delivering the exogenous DNA, a result that can be partially explained by incorrect folding of these ScFv-P-L fusion proteins (Fig 4). As a control, ScFv alone was unable to deliver exogenous DNA.

To further confirm that the ScFv fusion protein–mediated gene transfer is *via* ErbB2 molecules, excess amounts of MAb against ErbB2 (40 μg/mL) or ScFv specific for ErbB2 (80 μg/mL) were used for preincubation with the ErbB2(+) cells before the addition of ScFv-P-S/DNA or ScFv-P-L/DNA complexes. As is shown in Figure 6, panel B, preincubation of either MAb or ScFv efficiently prevented ScFv-P-S– or ScFv-P-L–mediated gene transfer, while as expected, pretreatment of cells with ErbB2-specific antibody (MAb or ScFv) shows no effect

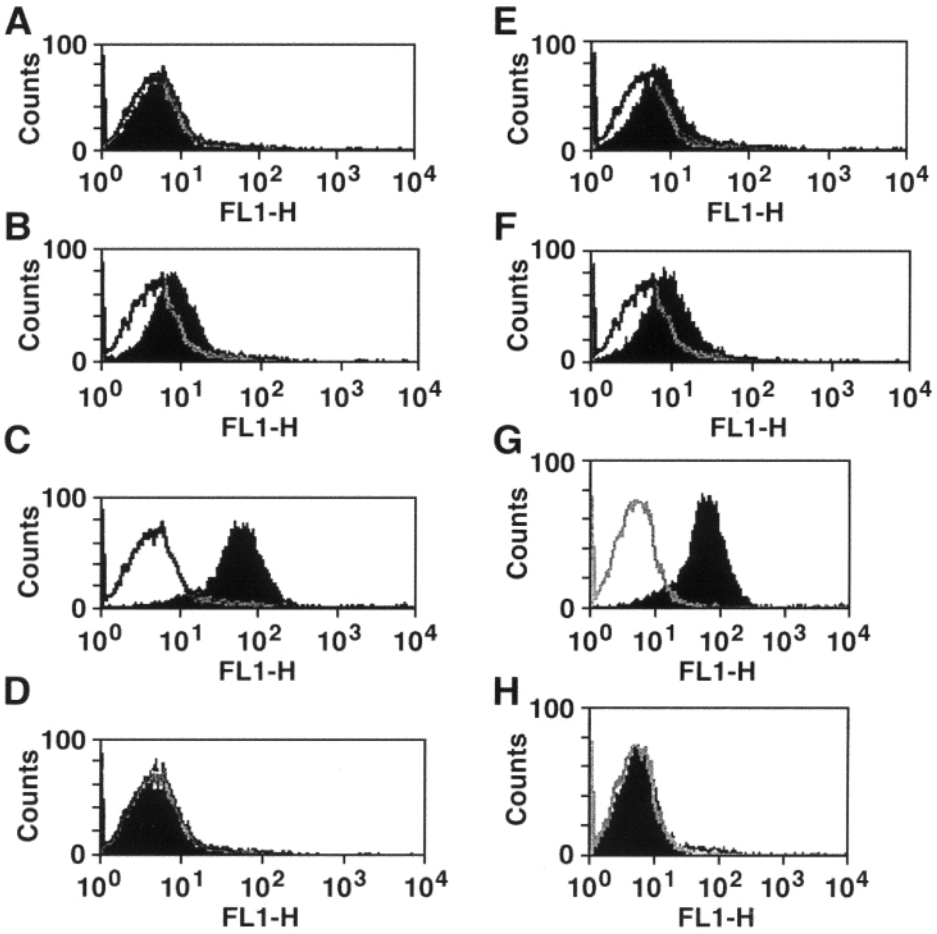


Figure 4. A refolding process is necessary to restore binding ability of the ScFv fusion proteins. Fusion proteins, either ScFv-P-S or ScFv-P-L, were refolded by dialysis sequentially with buffer I and buffer II (see *Materials and Methods*). Samples obtained from each of the steps were analyzed for their ability to bind to surface ErbB2 molecules on SKBR3 cells by FACS assay as described in Figure 3. **Panel A:** ScFv-P-S before dialysis. **Panel B:** ScFv-P-S after buffer I dialysis. **Panel C:** ScFv-P-S after buffer II dialysis. **Panel D:** ScFv-P-L before dialysis. **Panel E:** ScFv-P-L after buffer I dialysis. **Panel F:** ScFv-P-L after buffer II dialysis. **Panel G:** Positive control using MAb against ErbB2 (Neu 9G6; Santa Cruz Technologies). **Panel H:** Negative control in which no first antibody was present.

on calcium phosphate–mediated gene transfer. It is also noted that calcium phosphate–mediated gene transfer is more efficient than the fusion protein–mediated gene transfer. However, as is shown in Figure 6, panel C, although calcium phosphate–mediated gene transfer is more efficient, i.e., approximately 10-fold higher than ScFv-P-S and 70-fold higher than ScFv-P-L, this gene transfer approach, as well known, is nonspecific as both the ErbB2(+) SKBR3 and ErbB2(−) MCF7 cells were equally transfected, whereas transgene expression mediated by the fusion proteins in SKBR3 cells is approximately 40- to 50-fold higher than in MCF7 (Fig 6C), i.e., 70 *vs.* 1.5 U of luciferase activity. Another control ErbB2(−) cell line, HCC70,[25] was also tested and no specific gene transfer mediated by the fusion proteins was detected under these same experimental conditions (data not shown). Taken together, these data demonstrate that ScFv fusion proteins are able to selectively mediate gene transfer into the ErbB2(+) cells, and correct refolding of these proteins is critical to facilitate binding and uptake of the DNA/protein complex by the cells. Nevertheless, although selective, the relatively lower efficiency of gene transfer mediated by the fusion proteins (compared to calcium phosphate transfection) (Fig 6, panels B and C) prompted us to introduce cationic lipids into the vector (see below).

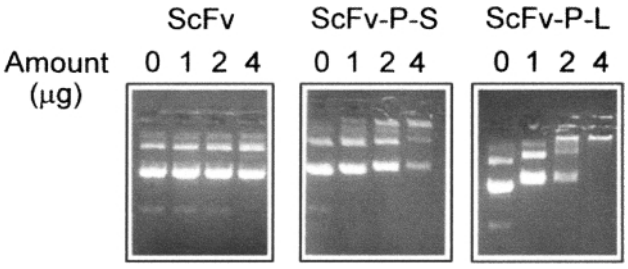
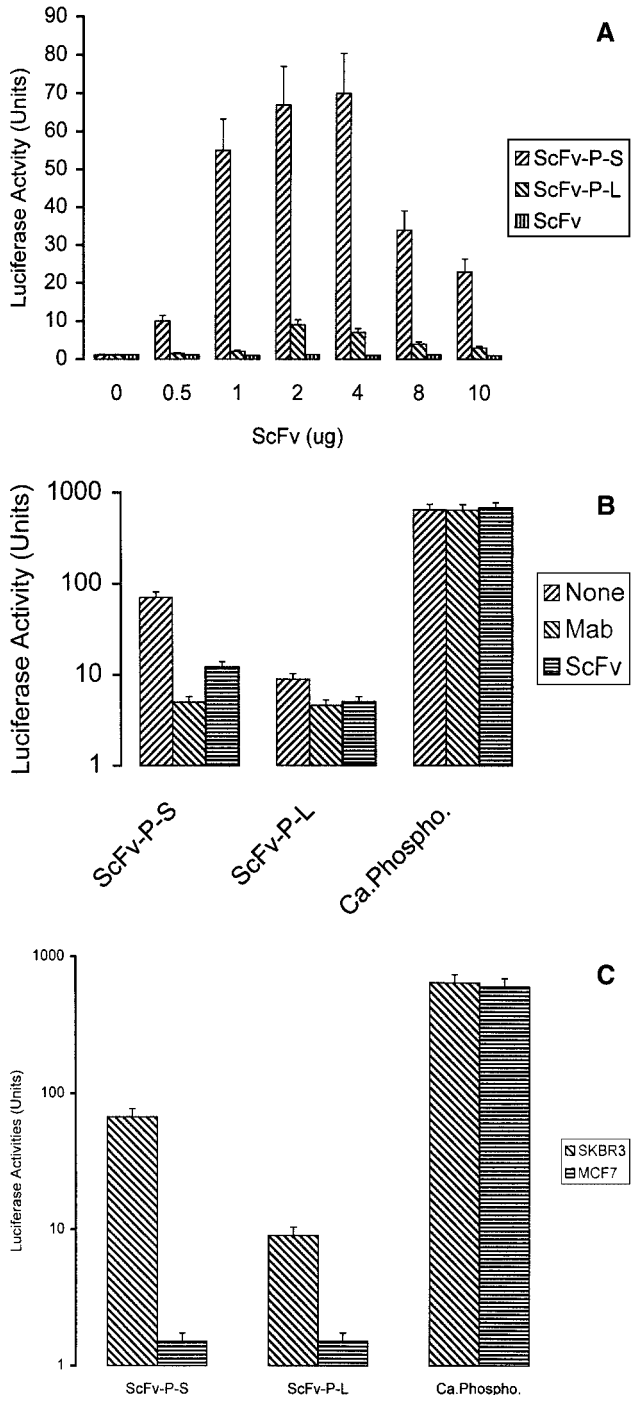


Figure 5. Ability of ScFv fusion proteins to bind with DNA. The ability of the ScFv fusion proteins to bind to DNA was analyzed in a gel shift assay. One microgram of pcDNA-Luc reporter was mixed with various amounts of ScFv, ScFv-P-S, or ScFv-P-L (as illustrated in the figure) and incubated at room temperature for 30 minutes. The mixtures were then loaded onto 1% agarose gel for analysis.

ScFv mediates selective gene delivery in the form of a DPSL complex

To achieve efficient and selective gene transfer, pcDNA-Luc plasmids (D) were complexed with three individual components including protamine (P), ScFv (S), and Dotap (L) as a complex designated DPSL in this report. We predicted by computer analysis that the isoelectric point of ScFv was approximately 6.7, suggesting that it could

statically interact with cationic lipids and protamine. To optimize conditions for complexing DNA, we mixed 0.1 μg of protamine, 1 μg of pcDNA-Luc, 4 μL of ScFv (1 μg/μL), and 20 μL of various types of lipids under several different conditions that mainly involved altering the order of the components added into the solution. As efficient gene transfer requires that DNA be protected from nuclease attack, complexes formed under various conditions were first tested for their ability to be resistant to DNase I treatment. To this end, excess amount of DNase I (40–80 U DNase I/μg of DNA) was used to treat the complexes at 37°C for 2 hours.



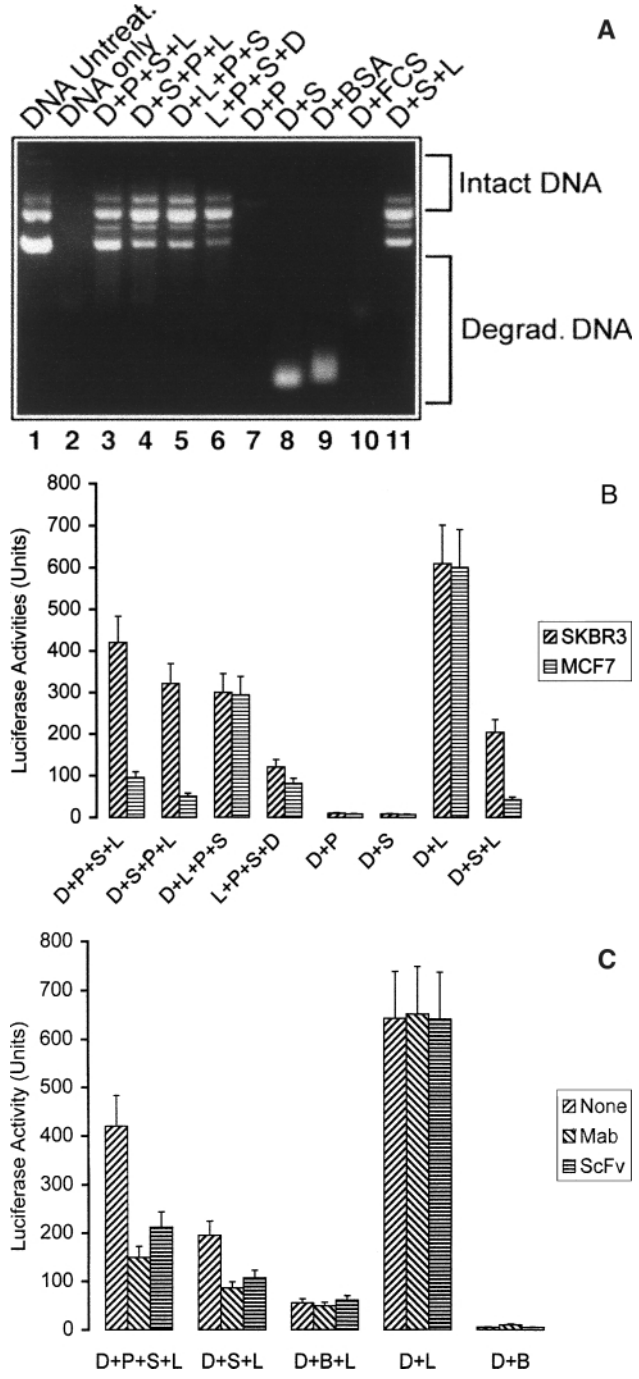
The reaction mixture was then treated with 1% SDS and Proteinase K at 1 mg/mL, followed by incubation at 37°C for 1 hour. Afterwards, the mixture was twice extracted with phenol/chloroform and the upper phase containing DNA was analyzed on 1% agarose gel. As is shown in Figure 7, panel A, if DNA (D) were added into the solution before Dotap (L) was mixed with ScFv (S) and protamine (P), DNA can be effectively protected against DNase I treatment (lanes 3, 4, 5). The intensity of DNA will slightly decrease when the DNA is added last (lane 6), suggesting that DNA may not be as well protected as in the cases in which DNA was added first (lanes 3, 4, and 5). Also in Figure 7, panel A, 0.1 μg of protamine alone shows little protection for the DNA (lane 7), while as expected, ScFv, FCS, and bovine serum albumin (BSA) provided no protection for the DNA against DNase I digest. There are also other controls that include: lane 1, no-treatment for the DNA and lane 2, naked DNA treated by DNase I alone. Clearly, Dotap itself can still protect DNA (lane 11). Taken together, DNA can be best protected if added prior to formation of a pre-complex consisting of S, P, and L.

Next we investigated whether DNA complexed under various conditions could be selectively delivered into ErbB2(+) cells. As shown in Figure 7, panel B, selective transgene expression was detected in SKBR3 cells, but not in MCF7 cells, treated with complexes (D+P+S+L) or (D+S+P+L), indicating that at least some, if not all, of the ScFv fragments were positioned on the surface or within the complexes in such a way as to function as the ligand. In contrast, both SKBR3 and MCF7 cells expressed similar levels of luciferase activity when treated with (D+L+P+S), a case in which DNA (D), protamine (P), and Dotap (L) were preincubated before the addition of ScFv, suggesting that the ScFv may not be correctly embedded in the complexes, and as a result fails to direct selective gene delivery into ErbB2(+) cells (see *Discussion*). If DNA was added last (L+P+S+D), transgene expression was low in efficiency. As expected, complexes (D+P) or (D+S) failed to deliver DNA into both types of cells. Interestingly, 0.1 μg of protamine appears to be necessary, as a complex without

Figure 6. Selective gene transfer mediated by fusion proteins with target specificity against ErbB2 receptor. **Panel A:** Transgene expression mediated by fusion protein (ScFv-P-S or ScFv-P-L). Various amounts of ScFv-P-S, ScFv-P-L, or ScFv (as a control) were mixed with 1 μg of pcDNA-Luc reporter at room temperature for 30 minutes. The mixtures were then added to ErbB2(+) SKBR3 cell culture (for details, see *Materials and Methods*) for a 48-hour incubation. Expression of luciferase activity was measured using a Promega luciferase assay kit. **Panel B:** Inhibition of fusion protein–mediated gene transfer by anti-ErbB2 MAb (40 μg/mL) (Santa Cruz Biotechnology) or ML39 ScFv (80 μg/mL). MAb or ScFv was preincubated with SKBR3 cells for 10 minutes before addition of fusion protein/DNA complexes to the cells. **Panel C:** Comparison of SKBR3 with MCF7 cells in transgene expression mediated by either fusion protein or calcium phosphate. pcDNA-Luc reporter–delivered fusion protein was the same as described above, while calcium phosphate–mediated gene delivery was performed using a Promega kit. The data represent duplicate samples that are usually tested three times. Error bars represent mean ± SD.

protamine (D+S+L) shows approximately 50% reduction in luciferase activity as compared to those cases in which protamine was present, i.e., in complexes (D+P+S+L) or (D+S+P+L). As a control, when Dotap alone was used to complex DNA (D+L), the highest transfection efficiency was observed, but transgene expression was nonselective.

To further confirm that gene delivery was mediated by ErbB2, excess MAb and ScFv (both against ErbB2) were preincubated with SKBR3 cells before the addition of the complexes. After incubation at 37°C for 48 hours, cells were lysed for analysis of luciferase activity. As is shown Figure 7, panel C, addition of MAb or ScFv resulted in approximately 50% inhibition of luciferase activity in cases of (D+P+S+L) and (D+S+L). As a control (D+B+L), BSA (B) was used in place of ScFv (D+S+L). In this case, BSA is clearly unable to facilitate selective gene delivery, but substantially interferes with general gene delivery as compared to the case of (D+L), in which the highest efficiency of gene delivery is obtained. As expected, neither MAb nor ScFv effected gene delivery in the cases of (D+B+L) or (D+L). Taken together, these results indicated that exogenous DNA can be selectively delivered into ErbB2(+) cells as DPSL complexes by mixing DNA (D), protamine (P), ScFv (S), and Dotap (L) together. Clearly, trace amount of protamine (0.1 μg), as predetermined by DNase protection assay, was able to improve transfection efficiency.



DISCUSSION

Targeted gene delivery into a defined cell population is of unquestionable importance for improving therapeutic efficacy and minimizing side effects derived from exogenous gene expression in tissues or organs not targeted for gene therapy. Whereas viral vectors have

Figure 7. Selective gene delivery mediated by ScFv in the form of a DPSL complex. **Panel A:** DNase I protection assay on complexes formed under various conditions. The complexes formulated under various conditions (see below) were treated by excess amount of DNase I (40–80 U of DNase I/μg DNA) for 2 hours at 37°C. The protein was then removed by Proteinase K treatment, followed by phenol/chloroform extraction, and subsequently analyzed by 1% agarose gel. Preparation of each of these complexes is as follows (for details, see *Materials and Methods*). **Lane 1**, DNA untreated control. **Lane 2**, DNA alone treated by DNase 1. **Lane 3**, (D+P+S+L) complex was prepared by preincubating DNA (D) with protamine (P) and ScFv (S) for 15 minutes, followed by addition of Dotap (L) for additional incubation of 15 minutes. The complex was then treated with DNase I. **Lane 4**, (D+S+P+L) complex was prepared by preincubating D with S and P for 15 minutes, followed by addition of L for further incubation of 15 minutes. The complex was then treated with DNase I. **Lane 5**, (D+L+P+S) complex was prepared by preincubating D with L and P for 15 minutes, followed by addition of S for further incubation of 15 minutes. The complex was then treated with DNase I. **Lane 6**, (L+P+S+D) complex was prepared by preincubating L with P and S for 15 minutes, followed by incubation with D for additional 15 minutes. P followed DNase I, then treated the complex treatment. **Lane 7**, (D+P) represents DNA mixed with protamine. **Lane 8**, (D+S) depicts DNA mixed with S, and treated by DNase I. **Lane 9**, (D+BSA) represents DNA mixed with BSA, followed by DNase I treatment. **Lane 10**, (D+FCS) depicts D mixed with FCS, followed by DNase I treatment. **Lane 11**, (D+S+L) denotes incubation of D with S and L, followed by DNase I treatment. **Panel B:** Transgene expression in either SKBR3 or MCF-7 cells transfected by various complexes as described in **Panel A**. Complexes prepared above **(Panel A)** were used for transfection of SKBR3 and MCF-7 cells, respectively. Luciferase activity was measured as described in legends of Figure 5. **Panel C:** Inhibition of transgene expression mediated by ScFv by MAb and ScFv. The experiments were performed as described in legends of Figure 6. Error bars represent mean ± SD.

proven to be extremely efficient in gene transfer, alteration in target specificity of the virus can result in a decrease in infectivity of the recombinant virus.[1,2,4–7] These findings, in addition to concerns about the safety and immunogenicity of the viral vectors, have prompted investigators to develop nonviral vectors with targeting capacities for selective gene delivery.[4–7,26]

A variety of liposomes and polymers have been developed and shown in recent years to be highly effective in gene transfer; however, most of these agents are nonspecific.[3,27,28] Targeting of nonviral gene delivery has been achieved by incorporating into the vectors ligands that can specifically recognize surface markers of the cells. Notably, transferrin incorporated into liposomes can substantially enhance efficiency of gene transfer mediated by the liposome.[29,30] Several groups, including our own, have used recombinant antibody fragments (Fab and ScFv) as ligands in the design of targeted nonviral gene transfer vectors.[13,14,19] The bifunctional nonviral vector we previously reported is of human origin, but difficulty in large-scale isolation of the Fab protamine fusion proteins was felt to restrict its further development.

To overcome the above problems, we conducted the current studies to evaluate two structural modifications in the targeted vectors including truncating the protamine portion of the human ScFv fusion protein to facilitate production of the protein and incorporating cationic lipids to improve transfection efficiency. Our results demonstrate that isolation of the ScFv fusion proteins remains problematic as neither of the fusion proteins was secreted into the supernatants, whereas a high level in expression of ScFv alone was obtained with a yield of 1–2 mg per liter of culture. Further analysis indicated that the ScFv fusion proteins could only be extracted from cell pellets by strong denaturants such as 6 M GuaHCl. The truncated form of fusion protein, denoted ScFv-P-S, was produced with a yield of 20 μg/L, whereas a 4-fold lower yield of the full-length form fusion protein (approximately 5 μg/L), ScFv-P-L, was obtained. In addition, an extensive refolding process was needed to recover binding activity. We found that ScFv-P-S was able to fully regain its ability to bind to ErbB2, but only a partial recovery was obtained for the full-length fusion protein, ScFv-P-L (Fig 4). Although both proteins were able to selectively deliver exogenous DNA into ErbB2(+) cells (Fig 6), ScFv-P-L was clearly less effective than ScFv-P-S, presumably as a result of misfolding of the ScFv portion of ScFv-P-L (Fig 4). These results indicate that when fused to the ML39 ScFv, this minimal region of human protamine, consisting mainly of arginine residues and without any of the cysteine residues found in full-length human protamine, is more easily produced and refolded. In addition, the truncated protamine polypeptide is sufficient in binding DNA molecules (Fig 5), and displays superior selective gene transfer capabilities as compared to the full-length fusion protein ScFv-P-L (Fig 6). A short stretch of positively charged amino acids capable of condensing DNA is not unprecedented; synthetic poly-L-lysine was successfully used to condense DNA in gene transfer studies.[31]

Although fusion proteins deliver exogenous genes in a selective way as confirmed by using MAb and ScFv as blocking reagents and ErbB2(−) cells as negative controls (Fig 6), the efficiency of transgene expression, as judged by absolute value of luciferase activity, is relatively low as compared with calcium phosphate–mediated nonspecific transfection, i.e., the latter is 20- to 100-fold more efficient than the former (Fig 6, panel C). Another weakness of the fusion protein system is that a lengthy purification and refolding procedure is needed for each fusion protein. These concerns, in addition to the fact that large quantities of ScFv itself can be easily obtained, prompted us to take a different approach to circumvent the aforementioned problems. To this end, we introduced the cationic lipid Dotap and prepared complexes consisting of four individual components including DNA, protamine, ScFv, and Dotap, denoted as DPSL. As expected, the slightly acidic ScFv and negatively charged DNA were found to be complexed well with positively charged protamine and Dotap, as demonstrated by resistance to nuclease and ability of selective gene delivery into ErbB2(+) SKBR3 cells (Fig 7, panel A). Interestingly, if DNA were added last to the preincubation mixture, (L+P+S+D), i.e., preincubation of Dotap (L), protamine (P), and ScFv (S) prior to addition of DNA (D), expression level of transgene was decreased (Fig 7, panel B). It is likely that DNA was not properly condensed as DNA appears to be inappropriately exposed to the outside of the liposome, as evidenced by an increased sensitivity to nuclease digestion. We also observed that selectivity of transgene delivery was lost when ScFv (S) was added last to the preformed complex, i.e., preincubation of DNA (D), Dotap (L), and protamine (P) prior to addition of ScFv as denoted (D+L+P+S) in Figure 7, panel B. Although highly resistant to nuclease (Fig 6, panel A), ScFv in (D+L+P+S) complex was presumably inappropriately embedded or even not attached to the complex, resulting in a loss of selectivity (Fig 7, panel B). The optimal condition was found to be preincubation of DNA (D) with protamine (P) in addition to ScFv (S) at room temperature for 15 minutes, followed by finally mixing with Dotap (L) as denoted (D+P+S+L).

In our studies, only 0.1 μg of protamine was used for preincubation with 1 μg of DNA. At this ratio between P and D, DNA was only partially condensed by protamine as demonstrated by the fact that this preformed P+D complex was highly accessible to nuclease (lane 7 of Fig 7, panel A). Nonsaturation of DNA by protamine appears to be essential because if excess amounts of P, i.e., 10 μg P:1 μg D, were used to preincubate with D before the addition of S and L, the complex lost its gene delivery capability (data not shown); most likely in this case, neither S nor L was able to form a complex with DNA. Whereas protamine itself was unable to replace lipids and ScFv in targeting gene expression, clearly, in our study, protamine (P) at appropriately low concentration can aid transgene expression. This is evidenced by the fact that complex minus protamine (D+S+L) displayed only 50% of luciferase activity of that in complex (D+P+S+L). Other types of lipids such as Dope, lipofectin, and LipofectAMINE were also compared with Dotap in our system and found to be lower in transfection efficiency (data not shown); however, a systematic investigation may be needed in future studies before a definitive conclusion is made. Other investigators

have also reported that protamine can improve transfection efficiency.[32,33] Whereas biochemical studies to date have not fully defined the exact mechanism(s) for protamine-mediated enhancement of gene transfer efficiency, its functional role in sperm, i.e., to condense DNA into a compact structure and deliver the DNA into the nucleus of the egg, in addition to its proven clinical safety, validates its potential use in future nonviral vector development.

Our results also highlight that the nonviral gene transfer systems reported here require substantial improvement. For example, whereas the fusion vector ScFv-P-S does display good selectivity with ErbB2(+) cells expressing approximately 8- to 10-fold more luciferase activity than the ErbB2(−) cells, it suffers from low efficiency of gene delivery, i.e., the absolute value of luciferase activity is about 10% of calcium phosphate–mediated transfection (Fig 6, panel B). Improvement measures such as incorporation of a moiety that facilitates DNA release from the endosomal or lysosomal compartments should be considered for future studies.[13] As far as the DPSL complex is concerned, our studies demonstrate an absolute luciferase activity approaching 60–70% of that mediated by lipids itself (Fig 7, panel B); however, it is not as selective as compared with the ScFv-P-S because only about 4- to 5-fold higher luciferase activity was observed in ErbB2(+) cells as compared to ErbB2(−) cells (Fig 7, panel B). Transduction efficiency as measured by percentage of LacZ(+) staining cells following treatment of DPSL demonstrated that approximately 75% of the ErbB2(+) cells was stained positive (β-Gal), whereas 50% of the ErbB2(−) was stained positive (data not shown). This poor selectivity implies that, in part, the ScFv is not adequately exposed on the surface of the complex to serve as ligand for interaction with the receptors of the target cells. Future studies should be aimed at improving selectivity by taking measures such as the incorporation of anchoring transmembrane domains capable of interacting with lipids so as to better "orient" the ScFv binding site on the surface of the complex.[34] In addition, exploration of new combinations of lipids or polycations should also be evaluated.[28,35–37]

Our study mainly differs from several previous reports in that a human ScFv was incorporated into the vector systems for target delivery.[13,29,31] The large repertoires of human antibodies that are currently available will allow selection of a broad range of ligands for targeting specific cell populations.[38–40] Whereas selectivity can be achieved for nonviral vectors formulated *in vitro* through combinations of DNA, ScFv, lipids, and protamine as demonstrated in this report, several important issues need to be carefully addressed for optimal targeting including the amounts and order of the components added into the mixture as well as the bioactivity and appropriate orientation of the ligands. Unquestionably, the performance characteristics of the current systems will also need to be validated *in vivo*.

In summary, we present a study that employs a human ScFv as ligand for targeted gene delivery. ScFv-P-S, a fusion protein consisting of a ScFv specific for ErbB2 and a truncated form of human protamine, is easier to be purified and refolded as compared to ScFv-P-L, a fusion protein consisting of the same ScFv but the full-length human protamine. ScFv-P-S displays high selectivity in delivering exogenous DNA *via* the ErbB2 receptor. We also present a different approach, which involves complexing DNA with three other individual components including protamine, ScFv, and Dotap, denoted DPSL. The DPSL was prepared by appropriately mixing the four components. The amounts and order of the components to be added into the mixture were guided by simple assays such as DNase protection and FACS analysis. The size and zeta potential of the particles, although not studied here, can be subsequently measured and the individual components of the particles adjusted to optimize *in vivo* delivery.[35] The DPSL complex demonstrates advantages over ScFv-P-S including ease in large-scale preparation in addition to higher transfection efficiency while substantially retaining target selectivity.

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