RESEARCH ARTICLE

Establishment of a HEK293T stable cell line capable of secreting GRP78-specific scFv-targeted nanovesicles

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ABSTRACT

Objective(s): Exosomes are nanovesicular vehicles capable of transporting different cargoes. Based on their characteristics, exosomes are proposed as a class of vehicles for targeted delivery of therapeutics. We aimed to establish a HEK293T stable cell line capable of secreting GRP78-specific scFv-targeted exosomes.

Methods: The pLEX-LAMP2b-GRP78 construct was developed by enzymatic replacement of DARPin in pLEX-LAMP2b-DARPin with a GRP78-specific scFv. pLEX-LAMP2b-GRP78 (or TurboGFP as control), psPAX2, and pMD2.G plasmids were co-transfected into HEK293T cells, and produced lentiviruses were harvested. Different multiplicities of infection (MOI; 10, 20, 30, 60, 120, and 240) were used for the transduction of HEK293T to select the most appropriate one as assessed by flow cytometry. Transduced HEK293T cells were subject to puromycin selection and the presence of the scFv was assessed in the established cell line at the DNA, transcript, and protein levels by PCR, RT-PCR, and Western blotting, respectively.

Results: pLEX-LAMP2b-GRP78 was successfully developed. Co-transfection resulted in the expression of GFP by HEK293T in the control group 48 hours following transfection. The MOI of 60 was selected as 10% of cells were GFP+ 72 hours following transduction. Following puromycin selection, the presence of the integrated scFv DNA and transcript was confirmed. Moreover, Western blotting results confirmed the presence of the His-tagged scFv in the established cell line.

Conclusions: HEK293T cells can be engineered for the production of targeted exosomes which could be applied for therapeutic purposes. Moreover, scFvs are potent targeting domains that could be leveraged for the development of targeted exosomes.

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INTRODUCTION

Extracellular vesicles (EVs) are particles secreted by various types of cells into the extracellular space. EVs are classified based on their size, function, biogenesis, and content. The main subtypes of EVs are exosomes, microvesicles (MVs), and apoptotic bodies [1]. Exosomes are small vesicles with a diameter ranging from 30 to 150 nm [2]. They are originated from endosomes and affect the acceptor cells by delivering proteins, lipids, and nucleic acids originating from the donor cells. Furthermore, exosomal membrane proteins interact with cells directly through ligand-receptor interactions or through their internal spilled-out contents and activate certain intracellular signaling pathways [3]. Exosome contents are protected from degradation by a unique phospholipid bilayer, which also harbors different kinds of proteins and lipids [4]. The ability of exosomes for acting as natural carriers has

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encouraged researchers to use them in drug delivery systems. In addition, compared with synthetic carriers, exosomes have several advantages such as low toxicity, high tissue and cell permeability, low immunogenicity, and biological barrier permeability [2]. The blood-brain barrier (BBB) is one of the main medical challenges in the treatment of central nervous system-involved oncological indications such as glioblastoma (GBM), and exosomes exhibit great potential to overcome this limitation [5]. Furthermore, the non-specific biodistribution of cargo is another challenge in the application of drug delivery systems [6]. In this regard, various exosome modification approaches have been investigated to address the mentioned challenge [6]. Of note, genetic engineering is one of the methods utilized for displaying targeting ligands fused with transmembrane proteins (such as LAMP2B) on the surface of exosomes [7].

GRP78 is a central member of endoplasmic reticulum (ER) chaperones, and it belongs to the HSP70 protein family. GRP78 is mainly present in the ER lumen and also in the mitochondria, nucleus, and cytosol [8]. This chaperone is a multifunctional protein that plays critical roles in protein folding and transporting misfolded proteins for ER-associated degradation (ERAD). In normal conditions, GRP78 binds to three ER transmembrane proteins acting as sensors. These proteins are activating transcription factor 6 (ATF6), protein kinase RNA-like endoplasmic reticulum kinase (PERK), and inositol-requiring enzyme 1 (IRE1) [9]. Under stress conditions such as in the tumor microenvironment (TME), misfolded proteins accumulate in the ER. In this situation, GRP78 binds to the misfolded proteins, and ATF6, PERK, and IRE1 sensors are released triggering different cellular pathways including overexpression of GRP78. The overexpressed GRP78 relocates to the cell surface, binds to different ligands, and triggers numerous cellular signaling pathways. Several studies have demonstrated that the expression of GRP78 increases in a variety of cancers, and there is a direct correlation between the expression level of GRP78 and increased levels of malignancy, chemoresistance, and metastasis [10]. The increased level of GRP78 expression on the surface of cancerous cells has paved the way for its targeting using different targeting platforms (for instance, antibodies) as a cancer treatment method [11]. Similar to different types of tumors, the overexpression of GRP78 in GBM has been reported in various studies [12].

Since GRP78 is overexpressed on malignant cells (while expressed at physiological levels by normal cells), it can serve as a target receptor to develop engineered GRP78-targeting exosomes that can be used as cancer drug delivery vehicles [11]. Herein, we developed a recombinant construct in a lentiviral vector in which a GRP78-specific scFv, previously developed in our laboratory, was replaced with DARPin fragments [11]. Moreover, lentiviral particles were harvested from HEK293T cells and subsequently used for the transduction of HEK293T cells as an attempt to establish a cell line proficient in the stable secretion of GRP78-specific scFv-targeted exosomes.

MATERIALS AND METHODS

Cells, plasmid construction, and reagents

The human embryonic kidney (HEK293T) cell line was used for viral packaging and stable cell line establishment by transduction in this study. This cell line was purchased from the Iranian Biological Resource Center (Tehran, Iran) and was cultured in Dulbecco's Modified Eagles Medium (DMEM; Sigma Aldrich, St Louis, MO, United States) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin (100 IU/mL) in a humidified condition at 37 °C (5% CO_2). The pLEX-LAMP2b-DARPin construct was re-engineered by replacing the DARPin fragment with the fragment encoding a GRP78-specific scFv, called "pLEX-LAMP2b-GRP78" [13]. Both the plasmid and the GRP78-specific scFv-encoding fragment were digested by NotI and XhoI restriction enzymes and were then ligated in the presence of T4 DNA ligase (Thermo Fisher Scientific, United States). Next, the resulting construct was transformed into *Escherichia coli* (*E. coli*) DH5α competent cells. The insertion was validated by colony PCR using a pair of primers specific for the scFv-encoding fragment and DNA sequencing. The pLEX-LAMP2b-GRP78 plasmid alongside psPAX2 and pMD2.G plasmids were used for the virus production step. Moreover, polyethylenimine (PEI; 25 kDa Mw; Cat. No. #9002-98-6, Sigma-Aldrich, Merck KGaA, Germany) transfection reagent was used as the plasmid co-transfection enhancer. All experiments were carried out according to the guidelines and the approval of *Tarbiat Modares University Research Ethics Committee.*

Lentiviral packaging

24 hours before co-transfection, HEK293T cells were seeded into 10 cm cell culture plates (4×10^6 cells) and were incubated at 37˚C in a humidified atmosphere (5% CO_2). After reaching the cell confluency rate of 80%, the medium was replaced with fresh serum-free DMEM, and the cells were incubated for an additional 2 hours in the incubator. The transfection solution was prepared using pLEX-LAMP2b-GRP78, psPAX2, and pMD2 plasmids, with a ratio of 2.1:2.1:1, respectively (21 µg, 21 µg, and 10 µg, respectively) mixed with PEI as the transfection reagent. Next, the transfection solution was gently added to the plates in a dropwise manner. Of note, the TurboGFP vector was used simultaneously to evaluate the co-transfection procedure (as the control GFP cell group). After 24 hours, the medium was replaced with fresh DMEM medium supplemented with 10% FBS. The expression of GFP by HEK293T cells was assessed 48 hours after the transfection step using fluorescence microscopy.

Virus production

The lentivirus-containing supernatant was harvested 36, 48, and 72 hours after transfection. The collected supernatant was centrifuged at 500 × g for 5 minutes to remove cells and cellular debris. Subsequently, the supernatant was centrifuged at 2000 × g for 20 minutes and the cell pellet was discarded. The harvested supernatant was filtered using 0.45 µm PVDF membranes. Collected viruses were concentrated by high-speed centrifugation at 19000 × g for 90 minutes and then were next stored at -80 °C until further use. The viral titer was determined using real-time PCR.

Multiplicity of infection (MOI), transduction, and stable cell line establishment

To generate a stable cell line capable of secreting exosomes with GRP78-specific scFvs on their surface, $2\text{-}3 \times 10^4$ HEK293T cells were seeded into the wells of a 24-well plate and were placed in an incubator overnight at 37 °C. After the cell confluency reached a rate of approximately 60%, the medium was replaced with fresh serum-free DMEM. After 2 hours, the virus-containing medium supplemented with 8 mg/mL polybrene was added to the cells at different multiplicities of infection (MOI; 10, 20, 30, 60, 120, and 240). On the next day, the medium was replaced with fresh DMEM containing 10% FBS. The efficiency of transduction was assessed by evaluating the percentage of GFP expression by GFP lentiviral particle-transduced HEK293T cells in each MOI group using the flow cytometry assay. The most favorable MOI was selected for the rest of the experiments as the expression of GFP by HEK293T cells transduced using this MOI was further validated under fluorescence microscopy 72 hours following transduction.

Stable cell line validation

The transduced cells were selected by adding an appropriate concentration of puromycin (0.5 µg/ml) to the medium one day after transduction and this pattern was repeated every two days for two weeks. Subsequently, total RNA was extracted from 5×10^5 transduced HEK293T cells using Qiagen's RNeasy Mini kit (Germany). The extracted RNA was quantified using a NanoDrop (Thermo Fisher Scientific, United States), and First-strand cDNA was synthesized using a commercial cDNA synthesis kit (Thermo Fisher Scientific, United States) according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed using a pair of primers specific for the GRP78-specific scFv-encoding DNA fragment to verify the cell line establishment step.

Confirmation of scFv-targeted exosome secretion by the established cell line using Western blotting

Since the established cell line was engineered to secrete exosomes targeted by His-tagged GRP78-specific scFvs on their surface, the expression of the His-tagged scFv can confirm that the established cell line is proficient in the production of targeted exosomes. To assess the expression of the His-tagged GRP78-specific scFvs by the transduced and untransduced HEK293T cells at the protein level, Western blotting was performed on the pellet of these cell groups. For this aim, anti-polyhistidine-peroxidase antibodies (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) were used.

RESULTS AND DISCUSSION

pLEX-LAMP2b-GRP78 construct development and transformation

The result of DNA sequencing validated the cloning step (data not shown). Moreover, Fig. 1 demonstrates the results of colony PCR confirmation assay on bacteria grown in selection media verifying the successful process of pLEX-LAMP2b-GRP78 construct cloning and transformation.

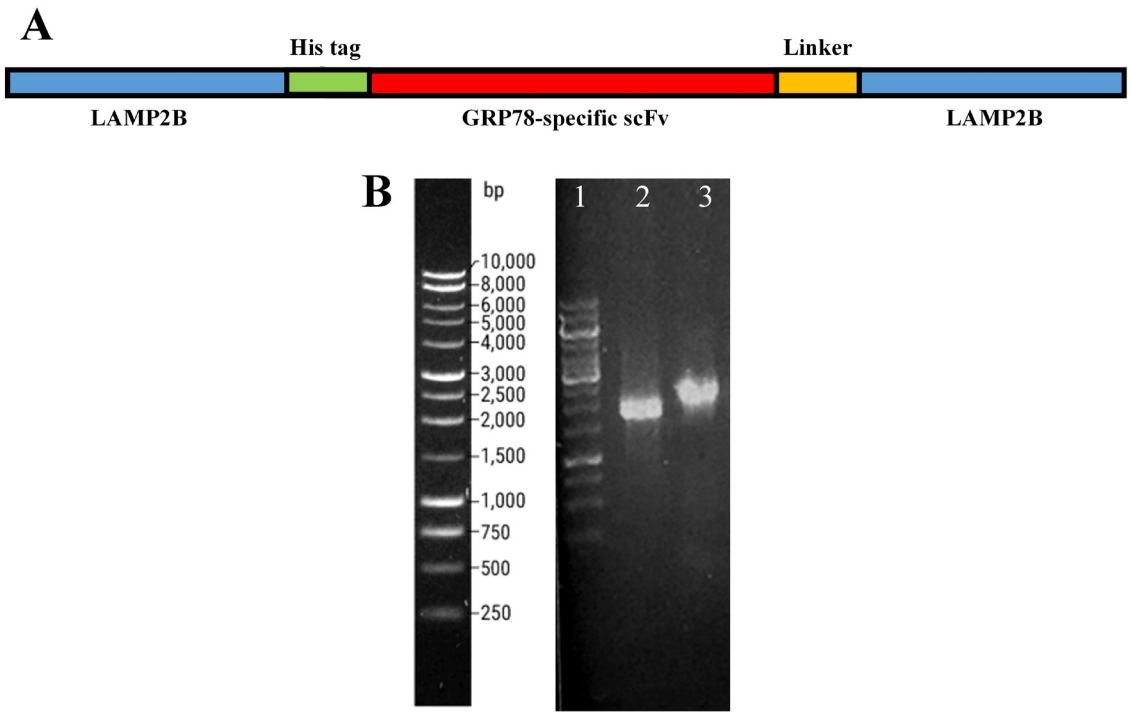

Fig. 1. pLEX-LAMP2b-GRP78 construct development, cloning, and transformation. A: pLEX-LAMP2b-GRP78 was developed by replacing the DARPin-encoding DNA fragment with that of the GRP78-specific scFv (using the restriction enzymes NotI and XhoI) in the pLEX-LAMP-DARPin construct. B: The pLEX-LAMP2b-GRP78 construct was transformed into DH5α competent bacterial cells, and colony PCR was performed to validate the cloning and transformation steps. Lane 1: 1 kb DNA marker, Lane 2: positive control, Lane 3: PCR amplicons of selection media-grown bacterial cells (around 1700 bp) electrophoresed on 1% agarose gel.

Transfection and transduction

The efficiency of transfection and transduction was evaluated using TurboGFP as a control vector. The percentage of GFP+ transfected HEK293T cells was estimated to be around 90% using fluorescence microscopy (Fig. 2). Viral titration was calculated to be 2×10^8 TdU/mL by quantitative real-time PCR (data not shown). Next, HEK293T cells were transduced with different MOIs (10, 20, 30, 60, 120, and 240). The rate of GFP-expressing cells after each round of transduction in each of the groups has been demonstrated in Fig. 3A. According to the flow cytometry analysis, different MOIs resulted in different percentages of GFP-expressing HEK293T cells ranging from 3% to 75%. The MOI of 60 was selected as the most efficient one as 10%

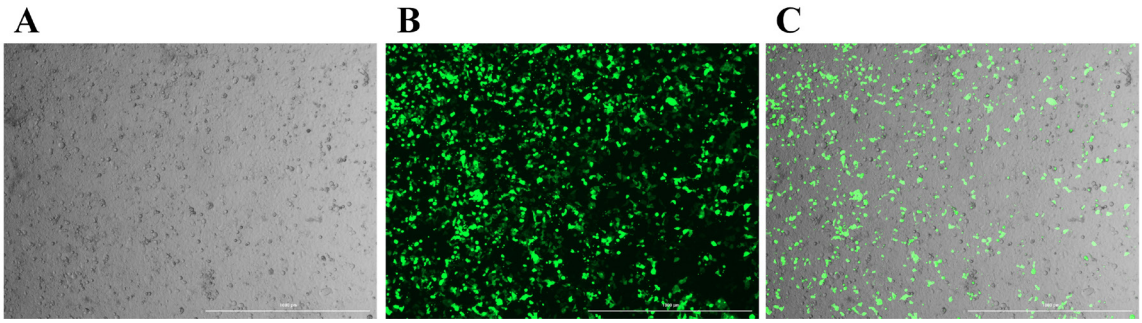

Fig. 2. Co-transfection of lentiviral vectors into HEK293T cells for the production of lentiviral particles. TurboGFP vector, psPAX2, and pMD2 plasmids were used for the co-transfection in the positive control group, and the expression of GFP was assessed 48 hours following transfection under fluorescence microscopy. A: bright field microscopic images, B: fluorescence microscopic images, C: merged microscopic images of HEK293T cells.

of the HEK293T cells were GFP-positive following transduction. The fluorescence and merged microscopic images of transduction with this MOI 72 hours following transduction are presented in Fig. 3B.

Stable cell line development

To select the transduced HEK293T cells stably secreting GRP78-specific scFv-targeted exosomes, the cells were screened throughout a puromycin (0.5 µg/mL) selection phase, and a puromycin kill cure was illustrated subsequently (Fig. 4A). The surviving cells were cultured and expanded eventually. Ultimately, the cell line establishment process was further validated by conventional PCR (at the DNA level) and RT-PCR (at the transcript level) as the presence of GRP78-specific scFv-encoding DNA fragments and transcripts, respectively, was confirmed by agarose gel electrophoresis (Fig. 4B).

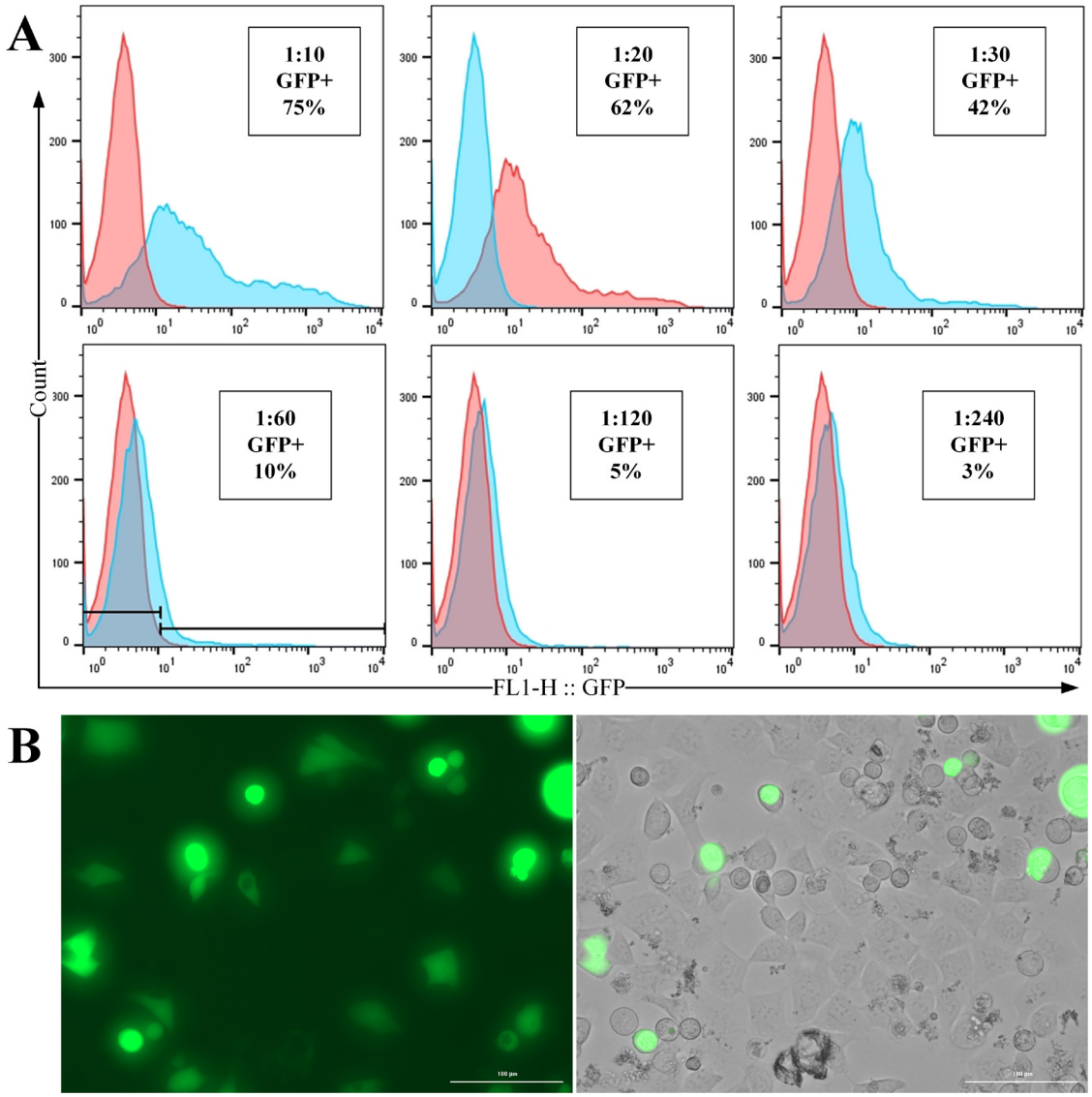

Fig. 3. Flow cytometric assessment for selecting the most efficient MOI for viral transduction and microscopic images of transduced HEK293T cells. A: Different MOIs (10, 20, 30, 60, 120, and 240) of GFP lentiviral particles were used for the transduction of HEK293T cells, and the MOI of 60 was selected for the rest of the experiments. B: Fluorescence microscopic images (left panel) and merged microscopic images (right panel) of HEK293T cells transduced with GFP lentiviral particles (MOI = 60) 72 hours following their transduction.

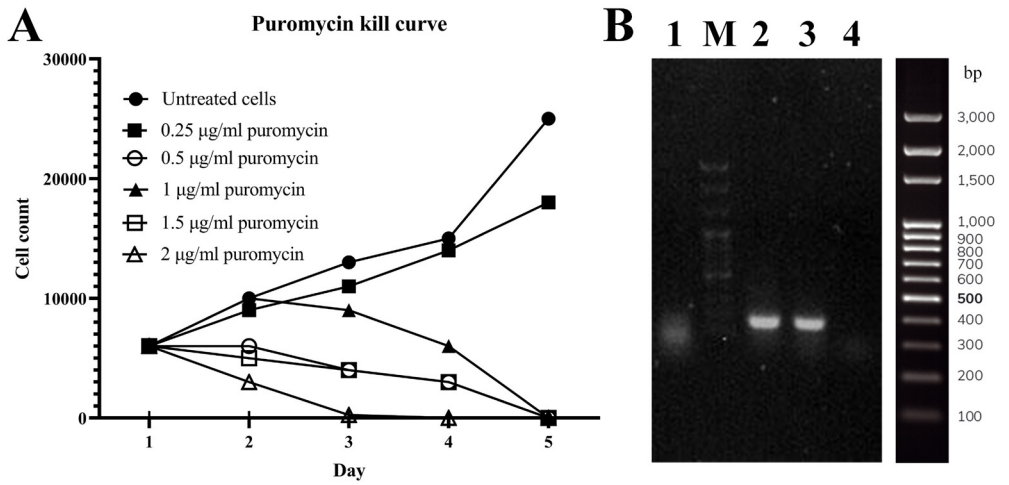

Fig. 4. Puromycin kill curve and PCR-based assessments for validating the presence of GRP78-specific scFv integrated DNA fragments and transcripts in the established cell line. A: Kill curve of puromycin for the selection of HEK293T cells transduced to stably a GRP78-specific scFv-targeted exosomes. B: Agarose gel electrophoresis of GRP78-specific scFv DNA fragments and transcripts. Lane 1: untransduced HEK293T cells at the DNA level, Lane M: 100 bp DNA marker, Lane 2: transduced HEK293T cells at the DNA level, Lane 3: transduced HEK293T cells at the transcript level, Lane 4: untransduced HEK293T cells at the transcript level.

Western blotting

Western blotting validated the expression of the His-tagged GRP78-specific scFvs in the established cell line, but not in the untransduced HEK293T cell group (Fig. 5). This means that the established cell line is capable of producing targeted exosomes with GRP78-specific scFvs on their surface which could be used for a variety of therapeutic purposes following careful preclinical assessments.

Recently, targeted cancer therapy has emerged to selectively target tumor cells without damaging healthy ones. However, selective targeting of cancer cells has remained a challenging aim to this day. For instance, various types of drug delivery vehicles have been investigated in this field for the targeted delivery of therapeutic agents to tumor sites. However, these delivery vehicles may come with disadvantages such as inappropriate biological properties including toxicity, high immunogenicity, and rapid clearance from the circulation [14]. In this regard, exosomes have been suggested for the same aim since they are natural cellular products with the ability to act as drug/cargo (such as nucleic acids and therapeutic drugs) carriers. Exosomes are vesicles with a nanoscale size that are secreted by almost all kinds of cells in the human body. These vesicles play important roles in intercellular communications [15, 16]. With such favorable biological properties, they can be used for efficient, safe, and targeted delivery of cargo. However,

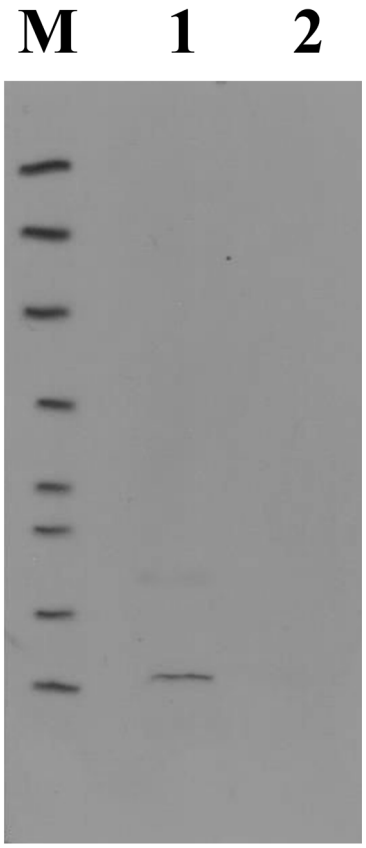

Fig. 5. Western blotting analysis assessing the expression of GRP78-specific scFvs on the surface exosomes. M, molecular marker, 1: Transduced HEK293T cells, 2: Untransduced HEK293T cells (control group).

exosome redirection to the desired tumor sites and cells is an important factor in this field. For this aim, various types of exosome redirection modalities have been proposed including aptamers, peptides, and antibodies [17-20]. Exosomes equipped with antibodies can specifically redirect cargo toward desired cell populations. For instance, Shi et al. genetically engineered cells to secrete exosomes displaying both anti-human epidermal growth factor receptor 2 (HER2) and anti-human CD3 antibodies [21]. These exosomes were capable of simultaneously targeting T-cell CD3 and breast cancer-associated HER2 receptors [21]. Such studies can highlight the preclinical feasibility of using exosomes for targeted cancer therapy.

GRP78 is a protein majorly engaged in the promotion of tumor outgrowth, metastasis, drug resistance, and apoptosis [22, 23]. This protein has specific tumor-associated mechanisms and is overexpressed in many types of malignancies including breast cancer, colorectal cancer, multiple myeloma, gastric cancer, lung cancer, and GBM [24, 25]. Such characteristics make GRP78 an ideal candidate for targeted cancer therapy using exosomes directed against this protein. In this regard, Shabani et al. used the ribosome display method to isolate an scFv against the C-terminal domain of a recombinant human GRP78 protein, termed CGRP [26]. In this study, we established a HEK293T stable cell line capable of secreting exosomes targeted with the mentioned GRP78-specific scFv. We first replaced the DARPin fragment in the "pLEX-LAMP-DARPin" plasmid construct with the fragment encoding the GRP78-specific scFv, and the generated construct was transformed into DH5α competent bacterial cells. Further, the reconstructed scFv-encoding plasmid was co-transfected into the HEK293T packaging cell line alongside other packaging and envelope lentiviral vectors to produce lentiviruses transferring the GRP78-specific scFv-encoding sequence. In the next step, the generated lentiviruses were used to transduce HEK293T cells making a stable cell line capable of secreting GRP78-specific scFv-targeted exosomes. To verify the establishment of the HEK293T stable cell line, we confirmed the scFv-encoding sequence integration in the genome of the established stable cells using PCR. Furthermore, we also used RT-PCR and validated GRP78-specific scFv expression at the transcription level. Ultimately, we confirmed GRP78-specific scFv expression at the protein level using Western blotting.

In the case of using antibodies for the targeting of exosomes, scFvs are more commonly used than full-length antibodies. This is because scFvs are generated by linking the variable heavy and light chain (V_H and V_L, respectively) of a monoclonal antibody (mAb) using a synthetic peptide linker. Therefore, scFvs harbor superior characteristics such as lower molecular weight, heterologous production, multimeric forms, and multivalency in comparison with full-length mAbs [27, 28]. Using more specific targeting modalities with enhanced functionality for exosomes can result in their improved cargo delivery capability to malignant cells and can further expand the applicability of these nanovesicles as natural therapeutics.

GRP78 was chosen because of its wide range of involvement in a variety of oncological indications including breast cancer, lung cancer, etc. [29]. This broadens the applicability of this target antigen for developing targeted therapeutics against a wide range of tumor cells proficient in the expression of GRP78 [29]. Targeting of exosomes with GRP78-specific scFvs can be used for the selective targeting of various types of cancer but it can specifically be beneficial in the case of GBM since BBB is a major challenge in terms of selective drug delivery to malignant cells. Exosomes can bypass this obstacle and selectively deliver their cargo to the brain tumor sites. The results of this study can serve as a pipeline for the production of scFv-targeted exosomes by generating stable cell lines. Such exosomes can also be loaded with different types of cargoes, including chemotherapeutics, miRNAs, siRNA, etc. for their targeted delivery specifically to the tumor cells. It is worth mentioning that both *in vitro* (using target cancer cell lines and primary cells) and *in vivo* studies using established xenograft preclinical mouse models are required for further verifying the herein presented data.

CONCLUSIONS

In this study, we employed a lentiviral gene delivery system for establishing a HEK293T cell line stably producing exosomes displaying GRP78-specific scFvs on their surface. These exosomes can be targeted toward cancer cells overexpressing GRP78 protein on the surface. To our knowledge, this is the first report on leveraging GRP78-specific scFvs for the selective targeting of exosomes. We are currently assessing the ability of these targeted exosomes to selectively target GRP78-proficient

cancer cell lines as well as patient-derived primary tumor cells. Moreover, investigating the targeting ability of these exosomes in preclinical animal models can further highlight their applicability.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest

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