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A gene-based anti-angiogenesis therapy as a novel strategy for cancer treatment

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ABSTRACT

Angiogenesis-targeted therapy of cancer is considered a promising strategy for therapeutic management of cancer progression. Over the last two decades, a few anti-angiogenesis monoclonal antibodies (mAbs) blocking VEGF signaling have been developed and approved by the FDA. The most widely used anti-angiogenesis drug is bevacizumab which binds VEGFA and prevents its interaction with VEGF receptor leading to suppression of angiogenesis. Despite the remarkable success in development of angiogenesis inhibitory mAbs, their clinical application is limited by the high-cost of mAbs-based regimen which includes multiple doses of mAbs due to their short biological half-life. Antibody gene therapy is an alternative system of antibody production. In this study, we have developed a gene-based anti-VEGF mAb system which is expected to produce a high concentration of anti-VEGFA mAb upon a single administration in cancer patients. The full-length cDNA bevacizumab light and heavy chains joint with T2A sequence were cloned in pCDH lentivirus vector. The lentiviral particles expressing bevacizumab was produced in HEK-293T cells. Recombinant lentiviral particles containing bevacizumab (rLV-bev) efficiently transduced HEK-293cells and produced functional bevacizumab mAb. Bevacizumab expression in the transduced cell was assessed by qRT-PCR and western blot at both the mRNA and protein level, respectively. The functionality of the recombinant bevacizumab was confirmed using the tube formation assay in the co-culture system of endothelial cells and HT-29cells transduced with rLV-bev viral particles. Our results show that rLV-bev gene therapy can be useful for angiogenesis-targeted therapy of cancer.

1. Introduction

Cancer angiogenesis is vital for cancer growth and progression. Therefore targeting angiogenesis is considered as a promising strategy for cancer treatment [1]. Angiogenesis rises from preexisting vessels and it is an important process in normal physiology [2]. In cancer, various types of cells and growth factors are involved in the tumor driven angiogenesis [3]. A well-known key factor for cancer angiogenesis is vascular endothelial growth factor (VEGF) which is valid target for cancer targeted therapy [4,5]. Bevacizumab (Avastin) is an FDA approved therapeutic monoclonal antibody (mAb) which binds to VEGF-A and blocks its interaction with VEGF receptor 2 (VEGFR2) leading to suppression of VEGF biological function [6].

Bevacizumab is used for the treatment of several types of human malignancies including colorectal cancer [6,7]. Bevacizumab is used as first-line therapy for metastatic colorectal cancer in combinations with chemotherapeutic agents including 5-fluorouracil (5-FU), oxaliplatin and capecitabine. Bevacizumab is also used for treatment of nonsmall cell lung cancer, neovascular age-related macular degeneration (NVAMD) and diabetic retinopathy (DR) [7,8]. Clinical studies show that the combination therapy of colorectal cancer patients with bevacizumab and chemotherapy or radiotherapy significantly improves their survival as compared with those treated with chemotherapy or radiotherapy alone [9,10]. These findings show that bevacizumab is a promising drug for cancer treatment.

Despite the success in development and clinical application of mAb

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for targeted therapy of cancer, the production and administration of therapeutic mAb are limited by the costly process of manufacturing these products. Besides, mAb has a fairly short biological half-life in the body and they have to be given to the patients in repeated doses, which in turn is costly for both patients and health care system [11,12]. Therefore in recent years, attempts have been made to develop gene/cell-based approaches for angiogenesis-targeted therapy of cancer [11]. In this, a viral or non-viral vector is used to deliver the coding sequences for mAb against angiogenesis promoting factor to cancer cells. The main advantages of a gene-based mAb system include, long-term sustained mAb expression, high serum mAb concentration, and no need for costly and sophisticated purification and preparation process [11,12].

Therapeutic efficacy of gene-based mAb systems for targeting angiogenesis has been shown in previous studies that have attempted to develop cell/gene based systems to suppress angiogenesis through the expression of anti-angiogenesis agents including angiostatin, endostatin and human soluble FMS-like tyrosine kinase receptor 1 (sFlt-1) [13,14]. In this study, full-length cDNA bevacizumab light and heavy chains joint with T2A sequence were cloned in pCDH lentivirus vector. Then we characterized the developed gene delivery system for the expression of biologically active bevacizumab in cancer cells.

2. Materials and methods

2.1. Cell culture

HEK-293T cells (Pasture Institute of Iran, Tehran, Iran) were cultured in Dulbecco's modified Eagle's medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA). The HEK-293T cells were detached with 0.25% trypsin, 0.03% EDTA at 37 °C, and sub-cultured every 1–2 days when they reach 70–80% confluency. HEK-293T cells were incubated at 37 °C in a humidified chamber with 5% CO2. HT-29 colon cancer cell lines were obtained from Pasteur Institute Cell Bank of Iran and cultured in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA). The attached HT-29 cells are sub-cultured when they reach 70–80% confluency using 0.25% (w/v) trypsin-0.53 mM EDTA (ATCC cat no. 30–2101). A split ratio of 1:10 to 1:12 or a seeding density between 4×10^4 and 5×10^4 viable cells/cm2 is used when sub-culturing HT-29 cells. HT-29 colon cancer was incubated at 37 °C in a humidified chamber with 5% CO2.

Primary Endothelial Cells (ECs) co-cultured with HT-29 colon cancer cells using the co-culture model system. Briefly, a frozen vial of EC cells was thawed in a 37 °C water bath, and then the recovered cells suspension were centrifuge for 3 min at 300 × *g*. The freezing medium was discarded and the cells were re-suspended in warm medium. The cells were seeded at concentration of 2.5×10^5 viable cells in 1 mL in T-75 culture flasks and incubated at 37 °C and 5% CO2. The culture medium was replaced by fresh media 24–36 h after seeding and every other day thereafter until the culture reached 80% confluency (4–6 days). ECs were utilized between passages 2 and 5. The transwell permeable supports (Corning, Tewksbury, MA) with a 1.0 μm polycarbonate membrane were used in the co-culture model system to separate ECs and HT-29 colon cancer cells into the different compartments [15]. Approximately 1.0×10^5 tumor cells (transduced and untransduced HT-29 cells) were layered on lower chamber. ECs were seeded in transwell insert (upper chamber) already coated by gelled basement membrane extract (BME) and EGM-2 media (Lonza, Swiss) was added to upper chamber. To prepare Transwell Chambers coated with Matrigel, BME was thawed at 4 °C and then diluted 0.5 × with cool BME dilution buffer or PBS pH 7.0. Transwell inserts were coated with 100 μl of 0.5 × diluted BME and the lower chamber (receiver) was coated with 300 μl of 0.5 × diluted BME. To solidify transwell chambers, the whole system were incubated at 37 °C and 5% CO2 for 30 min.

2.2. Design and synthesis of the expression construct

We considered a Kozak sequence (GCCACC) for optimal translation before IgE gene secretory signal peptide (sp) and the full-length antibody bevacizumab CDS. The light and heavy chains of bevacizumab synthesized in a frame all-in-one with a peptide sequence of Self-Cleavage (T2A peptide sequence) with TAA stop codon after light chain gene using Gibson Assembly kit (NEB, USA). Bevacizumab construct with XbaI and EcoRI restriction sites synthesized and cloned into a pUC57 vector (GenScript, USA). We subcloned bevacizumab construct from the pUC57 vector into the XbaI-EcoRI site of the pCDH513-B third generation lentiviral vector, including of the chimeric Rous sarcoma virus-long terminal repeat (RSV-5′LTR) promoter that leads to Tat-independent, 5′LTR-X-3′LTR RNAs transcription in Lentivector package process. Some right clones were purified by apply to miniprep plasmid kit (Thermofisher, USA). The right clones were confirmed by digestion and subsequent sequencing. About 300 ng plasmid was digested, at 37 °C water bath for 40 min and separated in 1% Agarose Gel. The pCDH513-B lentivector without bevacizumab construct was served as control (lentivector-non).

2.3. Generation and titration of lentiviral particles containing recombinant bevacizumab

Lentiviral particles were generated in HEK-293T cells using the pMD2.G, pRSV-Rev and pMDLg/pRRE packaging system by calcium phosphate (CaPo4) as described in Torono protocol for the production of recombinant lentivirus (rLV), a third generation system [16]. A total of 21 μg from transfer vector (lentivector-bev/lentivector-non), 7.5 μg pMD2.G vector, 15 μg pMDLg/pRRE vector, and 13 μg pRSV-Rev vector were dissolved in HEPES buffered water to reach 921 μl. After that, we added 33 μl Tris-EDTA (TE) buffer and then, mixed by the mixture strongly and left for 3 min at room temperature (RT). We added 105 μl $CaCl_2$ 2.5 M to the mixture and vortexed strongly [16]. For making DNA-$CaCl_2$ interaction; the mixture was vortexed while adding 1074 μl HEPES 2X. Lastly, we added 2100 μl master mix per 10 cm HEK-293T cells with 70% confluency. The transfection medium was replaced with 13 ml fresh medium containing 10% FBS, after 15–17 h incubation. We determined the transfections rate by counting GFP positive and negative cells under a fluorescent microscope [16].

The media of HEK-293T cells containing constructs with recombinant lentivirus bevacizumab (rLV-bev) and recombinant lentivirus without recombinant bevacizumab (rLV-non) was collected at 48 and 72 h after transfection. The collected media was centrifuged at 3000 × *g* for 15 min, filtered through a 0.2-μm filter, and freshly used for transduction. We used polyethylene glycol (PEG) method to measure recombinant lentiviral concentration. We added PEG 600 50%, NaCl4 M, and PBS to pooled recombinant viruses inside polypropylene bottles. Then, the bottles were mixed every 30 min and stored at 4 °C for 1.5 h. After that, tubes were centrifuged at 7000 g for 15 min at 4 °C. Based on Trono Lab protocols, the recombinant lentiviral titration was done by WPRE primers: F: 5′ACTGTGTTTGCTGACGCAAC3′ R: 5′CAA CACCACGGAATTGTCAG3′ and quantitative polymerase chain reaction (qPCR). We used volumes of 1000, 500, 100, 50, 20 and 0 μl of fresh viruses for transducing HEK-293T cells in a 12-well plate. Concentrated viruses were used including volumes of 4, 2, 1, 10^{-1}, 10^{-2} and 0 μl.

2.4. The gene expression studies

We assessed gene expression of bevacizumab construct by quantitative polymerase chain reaction (qPCR). HEK-293T cells were transduced with the rLV-bev particles and then they were selected with 2 μg/ml puromycin. The transfection efficiency detected by copGFP marker expression under the fluorescent microscope revealed that approximately 90–95% of HEK-293 T cells were transduced with the rLV-bev particles. Total RNA was extracted from the transduced HEK-293T in a

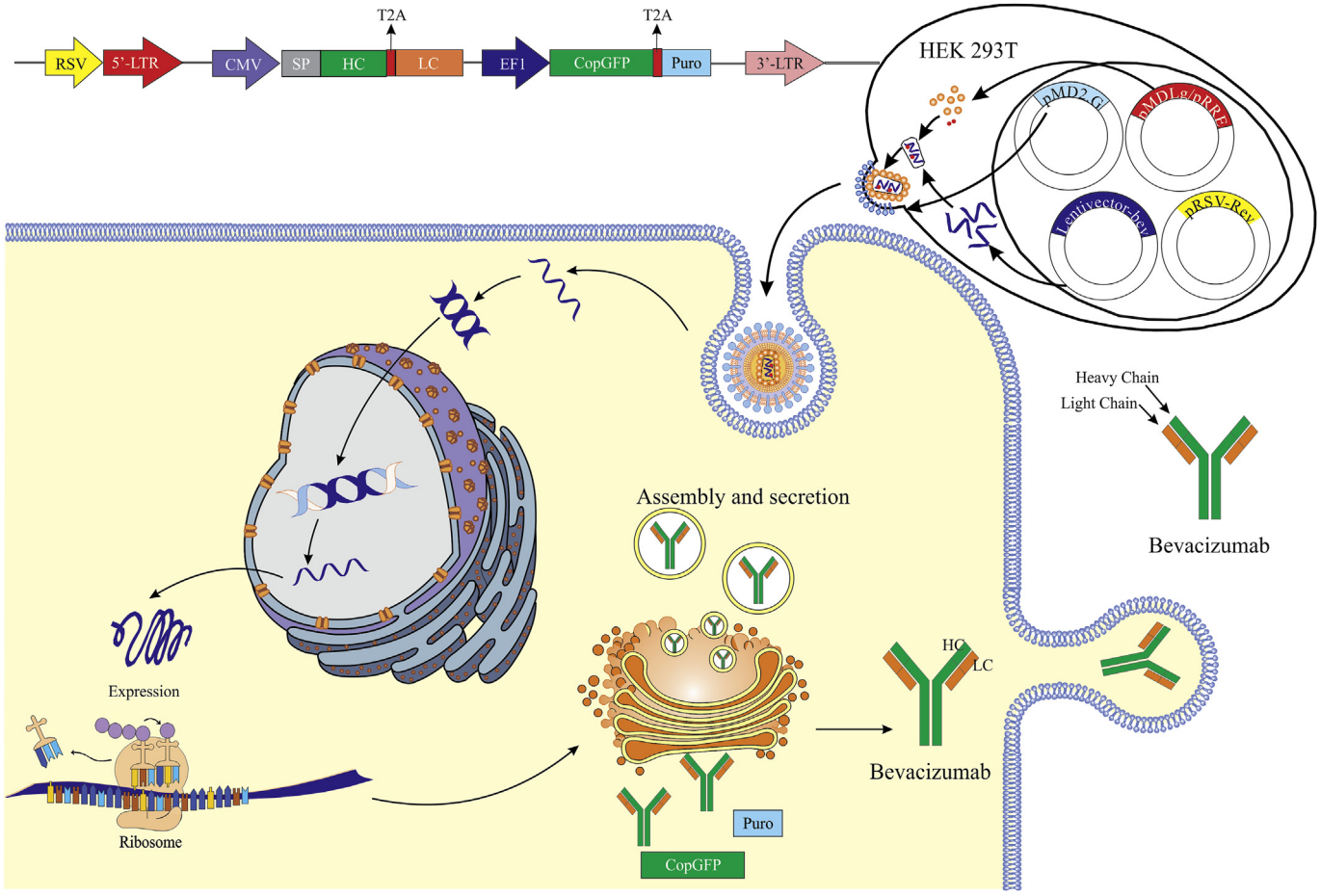

Fig. 1. Polycistronic lentiviral vector, Transfection and Expression of Constructs: The RSV promoter Tat-independent before 5′-LTR is essential for viral packaging process. Two promoters including CMV and EF1 for transcription of Bevacizumab gene and selection marker respectively. The "self-cleaving" 2A peptides have been used mediate protein cleavage from a single open reading frame through a ribosomal skip mechanism. For specific cleavage and elimination of T2A peptides, furin cleavage (FC) sites were included before T2A. Lentiviral vector packaged in HEK-293T cells using the pMD2.G, pRSV-Rev and pMDLg/pRRE packaging system by calcium phosphate. This polycistronic lentiviral overexpression four protein simultaneously in transduced cells. The antibody of Bevacizumab secreted from the cell because of secretory signal peptide.
RSV; Rous sarcoma virus, LTR; Long terminal repeat, CMV; Cytomegalovirus, EF1; Elongation factor-1, SP; IgE gene secretory signal peptide and T2A; Thoseaasigna virus 2A.

single well of a 6-well plate using the mRNA extraction kit (Qiagen, Germany) according to manufacturer's protocol. Real-time PCR was carried out using 0.5 μg RNA with SYBR Green. Primers used for qPCR are gene-specific Forward Primer F -5′ GATAAAACTCATACCTGCCC (10 μM) 2 μl, and gene-specific Reverse Primer R-5′ AAATGGTCAGAG TAAAGTCAG (10 μM) 2 μl. Data were presented as the ratio of mean threshold targeted human exogenous genes expression to human endogenous GAPDH, using the $2^{-\Delta\Delta Ct}$ method. The specificity of the PCR product was assessed by verifying a single peak on the plots obtained from the melting curve analysis [17]. The expression of bevacizumab mAb in HEK-293T cells was assessed by Western blot analysis. Supernatant of transduced HEK-293T cells (collected after 72 h) were analyzed for protein concentration using BCA protein assay kit (Thermo Fisher, USA). 30 μg protein from the concentrated lysate total protein from of rLV-bev, and rLV-non transduced HEK-293T cells was loaded in each lane onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Protein Ladder (Bio-Rad, USA) [18]. The antibody was also purified from the supernatant using protein A purification column and loaded in the gel as a positive control. Proteins in the polyacrylamide gel were transferred to the nitrocellulose membrane. Bevacizumab proteins were detected using rabbit anti-human IgG antibody (Abcam, ab6785) as primary antibody and, a secondary horseradish peroxidase (HRP) antibody goat anti-rabbit (Abcam, UK). Finally, the protein bands were visualized by exposure on X-ray film after the membranes were treated with enhanced chemiluminescent reagent A (luminol-based) and B (chemiluminescent substrate oxidizing agent) solution.

2.5. Transduction of HT-29 cells and MTT cell proliferation assay

The HT-29 cells were cultured at the confluency of 40–50% in a 6-well plate. The rLV-bev and rLV-non viral particles were carried out in multiple of infections (MOI) 5–10 for HT-29 cells transduction. The rLV-bev transduced HT29 cells were selected using puromycin (1.5 μg/ml), 72 h following transduction and they were also analyzed for transfection efficiency using fluorescent microscope.

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was performed to measure the viability of non-transduced HT-29 cells compared to that of HT-29 transduced with rLV-bev particles [19]. To this end, 10 μL of MTT solutions (Sigma-Aldrich, U.S.A.) were added to each well of a 96-well plate tissue culture plate, containing 8×10^3 cells and incubated at 37 °C for 24 h [20]. The cells were washed twice with 0.9% saline and then added with 200ul DMSO [20,21]. The absorbance (A) was identified at a range of 570 nm as a reference using a plate reader (BioTek Instruments, United States) [21]. All MTT assays were repeated three times. The cell number was

calculated from the value of OD570 according to a standard curve.

2.6. Angiogenesis assay by tube formation test

We used tube formation assay for evaluating angiogenesis in vitro. The rLV-bev transduced HT-29 cells were co-cultured with EC in the 96-well culture plates. HT-29 transduced with rLV-non particles and non-transduced HT-29 were also co-cultured with EC as control groups [15,22]. Tube area was measured by tube length image. To calculate EC tube length formation, each well of the 96-well culture plates was calculated, by the 3 images each well [22]. Images were analyzed for tube length formation. Analysis of phase contrast microscope image was measured using microscope camera software (ISCapture) by selecting ' measure' and then "line tool". This software was to calculate the distance between two random points or lines in the image (1 pixel = 0.34 μm). The procedure was repeated for all the tube lines in the image. After measuring every tube in the picture, data were entered into Excel. After normalization and standardization of the data, the average tube length (μm) of each well and the average tube length for all wells were determined and used to calculate the length of average tube length for three groups of transduced and non-transduced HT-29 cells co-cultured with EC.

3. Results

3.1. Construction of recombinant lentivector (transfer vector)

Recombinant lentivector containing bevacizumab heavy chain (HC) coding sequence and light chain (LC) coding sequence was constructed using DNA assembling, Gibson assembly method. The construct rLV-bev pCDH-CMV-heavy-T2A-light-EF1-copGFP-T2A-puro was verified by digestion and subsequent sequencing. The self-cleaving T2A peptide was also included to ensure complete release of final produced HC and LC antibody independent. The bevacizumab gene was placed under the control of the strong human cytomegalovirus promoter (CMV) for efficient expression in mammalian cells. Green fluorescent protein (copGFP) was linked with T2A peptide to puromycin under the control of EF1 promoter (System Biosciences, USA) (Fig. 1).

3.2. Production and titration of rLV-bev lentiviral particles in HEK-293T cells

rLV-bev and control rLV-non viral particles were produced in HEK-293T cells using the CaPO4 method. Viral plaque became visible at 24 h after transfection (Fig. 2a). After purification of viral plaque, rLV-bev viral particles were identified and titrated using quantitative PCR (qPCR). rLV-bev viral particles titration was found to be 1.2–2.4 $\times 10^6$ particles/ml. We used titration 1.5–3 $\times 10^7$ infection-unit/ml. The rLV-bev viral particles concentration was estimated using PEG 6000. High-efficiency packaging recombinant lentiviral (MOI 5–10) does was selected. HEK-293T cells transduced with rLV-bev were selected by puromycin (1.5 μg/ml). Transfection efficacy was confirmed by fluorescent microscopy 80–90% (Fig. 2a).

3.3. Bevacizumab expression in HEK-293 T cells

Transduction of HEK-293 T cells by recombinant LV-bev was confirmed GFP protein expression detected by a fluorescent microscope (Fig. 2b). We used qPCR and Western blotting to detect the transcript and protein level of bevacizumab expressed by the transduced HEK-293 T cells, respectively. As it is shown in Fig. 2c, bevacizumab transcript was detected in HEK-293T cells transduced with rLV-bev particles. Western blotting confirmed bevacizumab expression in rLV-bev transduced HEK-293 T cells at the protein level. These results confirm bevacizumab antibody is correctly transcribed and translated by the designed rLV-bev, as kappa-IGg1 is detectable by anti-human IgG1 Fc antibody (Fig. 2d). Western blotting analysis showed similar expression levels for GAPDH protein in both control and transduced cells in comparison with bevacizumab proteins that were only expressed in transduced cells (Fig. 2d). Bevacizumab purified from the cell supernatant using purification column is shown as A2 (Avastin2) and A1 (Avastin1) is concentrated lysate total protein from of rLV-bev transduced HEK-293T cells. (Fig. 2d).

3.4. Expression of bevacizumab and cytotoxic effects of rLV-bev transfection in HT-29

The HT-29 colon cancer cells were transduced by rLV-bev viral particles and the transduced cells were selected by puromycin. HT-29 cells transduction rate was confirmed by observation of GFP under a fluorescent microscope after 72 h (Fig. 3a). The cytotoxic effects of recombinant lentiviruses in HT-29 cells was assessed by MTT assay. As shown in Fig. 3b, transduced of HT-29 cells with rLV-bev didn't result in significant loss of cell viability in these cells ($P > 0.05$). These assay results that were carried out by the same MOI showed transduced and untransduced HT-29 cells had no significant differences in terms of cell viability ($P > 0.05$).

3.5. Functional analysis of recombinant bevacizumab by tube formation assay

RLV-bev transduced HT-29 cells were co-cultured with EC cells and tube length formation in the co-culture was compared with what observed in the co-culture of HT-29 cells and rLV-non/non-transduced cells as controls. Tube length formation has been used to assess the biological activity of anti-angiogenesis drugs in vitro. Scoring of the counting tube length is a valid method to quantitate the assay and get an accurate assessment of biological activity of anti-angiogenesis drugs such as bevacizumab. Co-culture of EC with HT-29 cells transduced with rLV-bev, resulted in a prominent inhibition of endothelial cell tubules, branch point formation, and the disruption of the tubular networks at 24 h. Fig. 3C shows tube length formation in the co-culture of EC cells with HT-29 cells transduced by either rLV-bev and rLV-non. Tubes began to form within 2–3 h of co-culture and reach to a peak at 5–6 h. Fig. 3 D shows the average tube length formation in each group, which was measured using the microscope camera image analysis and statistically analyzed by Microsoft Excel diagram (Fig. 3D). The value for average tube length was found to be 988.77 μm, 977.1 μm, and 580.36 μm for control co-culture, the co-culture of HT-29 rLV-non with EC, and the co-culture of HT-29 rLV-bev with EC, respectively. These findings show that the average tube length significantly decreases in HT-29 rLV-bev co-cultured with EC as compared with what observed in the co-culture of HT-29 rLV-non cells and untransduced cells.

4. Discussion

Here we report the development of an anti-angiogenesis gene-based therapeutic system which produce bevacizumab, a widely used anti-angiogenesis mAb. Given the biological significance of angiogenesis in cancer progression, efficient blocking of angiogenesis is considered a promising strategy for cancer treatment. The clinical administration of the approved anti-angiogenesis mAbs is costly as they have a short biological half-life and they have to be given in multiple doses. A gene-based anti-angiogenesis mAb method is expected to be more efficient and cost-effective for cancer treatment. Thus we attempted to develop bevacizumab producing viral vectors in the current project and characterize them for production of functional mAb and inhibition of angiogenesis in a colon cancer model in vitro. Metastatic colorectal cancer is characterized by neo-angiogenesis and it is considered to be an appropriate model for assessing the efficacy of angiogenesis-targeted therapeutic approach [23].

Our developed gene delivery system produce functional

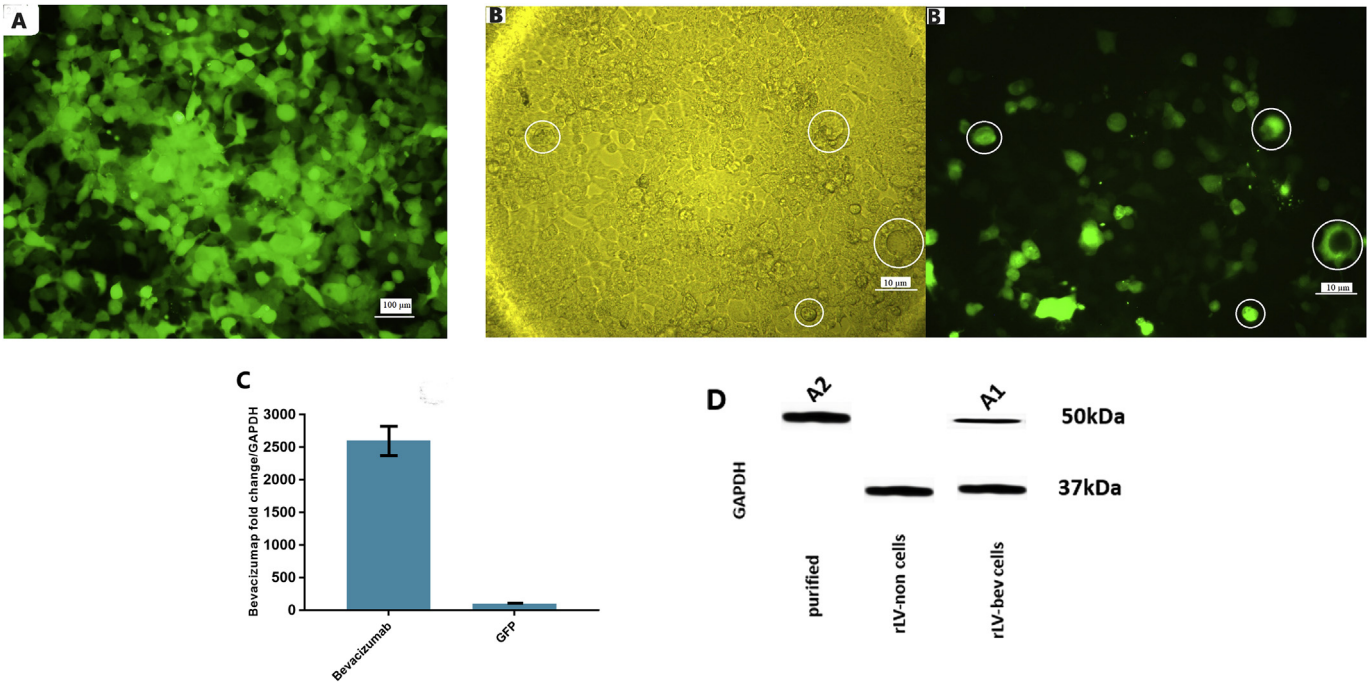

Fig. 2. Expression of bevacizumab in HEK-293T cells transduced by rLV-bev lentiviral particles. A. Transfection of HEK-293T cells using the CaPO4 method (magnification: 100×). B. bevacizumab expression was detected by the expression of copaGFP under a fluorescent microscope. The cells under open light and fluorescent microscope are shown from left to right, respectively (magnification: 100×). C. Real-time PCR. qPCR showed the expression of the genes at the mRNA level in comparison with endogens GPDH gene ($P < 0.05$). Cells transduced with rLV-bev or GFP-only recombinant lentiviruses are shown as rLV-bev and rLV-non cells, respectively D. Western blot analysis of rLV-bev expression. 293 cells were transduced with rLV-bev encoding bevacizumab (Avastin,A) and after 72 h, the supernatant was analyzed by Western blotting using a monoclonal antibody against IgG1 of bevacizumab. A2 is purified bevacizumab.

bevacizumab, a mAb against VEGF-A. Clinically used bevacizumab is an IgG1 antibody frame that includes two same light chains and two heavy chains with the molecular weight of 150 kDa (Roche, Data Sheet). Similar to other mAbs, bevacizumab has a short biological half-life and it is given in repeated doses to the patients, resulting in a costly treatment regimen for cancer patients [11]. In this work, development of lentiviral vectors expressing bevacizumab was pursued as an approach for production of a cost-effective and efficient anti-angiogenesis treatment regimen. The main challenge for the expression of mAb by gene-delivery system is the production of biologically active protein which can efficiently interact with its potential target and exert the desired biological effects [24]. Besides the expression of a full-length bevacizumab mAb in a single vector, is limited by vector capacity, therefore, the coding sequence for heavy and light chain might be needed to cloned in separate vectors. The main problem with cloning the heavy and light chain in separate vectors is their uncoordinated and non-simultaneous expression, which results in production of a non-functional mAb. To overcome this obstacle, we introduced T2A peptide linker which was placed between the coding sequence for heavy and light chain in a single vector with a promoter (CMV) and a terminator. The toxic effects of imbalance expressions of the heavy and light chain are internal ribosome entry site (IRES) bicistronic barrier. In this study, imbalance expression solved by T2A self cleavage peptide as bevacizumab, T2A, and Furin DNA sequences were cloned after CMV promoter in a single open reading frame (ORF). T2A peptide was also used for co-expression of copGFP and puromycin resistance gene under control of EF1 promoter. Lentirvial particles containing the designed construct was produced in lentiviral vecotrs which are considered to be very potent gene delivery systems.

Our designed lentiviral construct expressing bevacizumab is expected to induce more potent anticancer effects as compared with what could be achieved by administration of bevacizumab protein drug. In support of this notion, it has been reported that the antitumor effects of a single dose DNA-based anti-HER2 mAb is almost the same as that of four doses (10 mg/kg − 1) herceptin injected intravenously [25]. Our designed system is expected to produce higher level of bevacizumab in comparison with DNA-based anti-HER2 mAb system as we have used CMV promoter in our construct and integrated lentiviral system. lentiviral construct has CMV promoter and integrated into the genome then it is expected to produce the long-term expression of bevacizumab in the transduced cells leading to some side effects reported for bevacizumab mAb. An important strategy to reduce the side effects of bevacizumab-encoding viral particles is targeting them to cancer cells such that the production of bevacizumab is limited to cancer cells only. As production of bevacizumab by cancer cells is expected to suppress cancer cells growth, remission of tumor will ultimately leads to termination of bevacizumab production and its potential side effects in the body.

In another study, the full-length herceptin mAb was expressed via gutless adenoviral vector and used in an episomal form which resulted in haptotropism, high transduction efficiency, and persistent expression [26]. Nevertheless, the induction of strong humoral and cellular immune response against adenoviral proteins restricted the repetitive administration of the developed system [26,27]. Adenovirus associated viral vectors (AAV)-mediated transfer of bevacizumab to the pleura is another method used for expression of bevacizumab. AAV vectors were found to support long-term transgene expression and lower toxicity as compared with adenovirus vectors [28]. The main disadvantage of AAV is small size and limited capacity for transfer gene. As we needed to clone copGFP and puromycin resistance gene in addition to the gene encoding the light and heavy chain of bevacizumab, we selected lentivector third generation system. Besides lentiviral vectors are more suitable for large-scale production and they do not induce strong anti-vector humoral and cellular immune response. KYMRIAH (tisagenlecleucel), an FDA approved gene therapy product, has been produced using a lentiviral vector [29].

Our findings show that our designed construct expresses recombinant bevacizumab full-length mAb which is biofunctionally

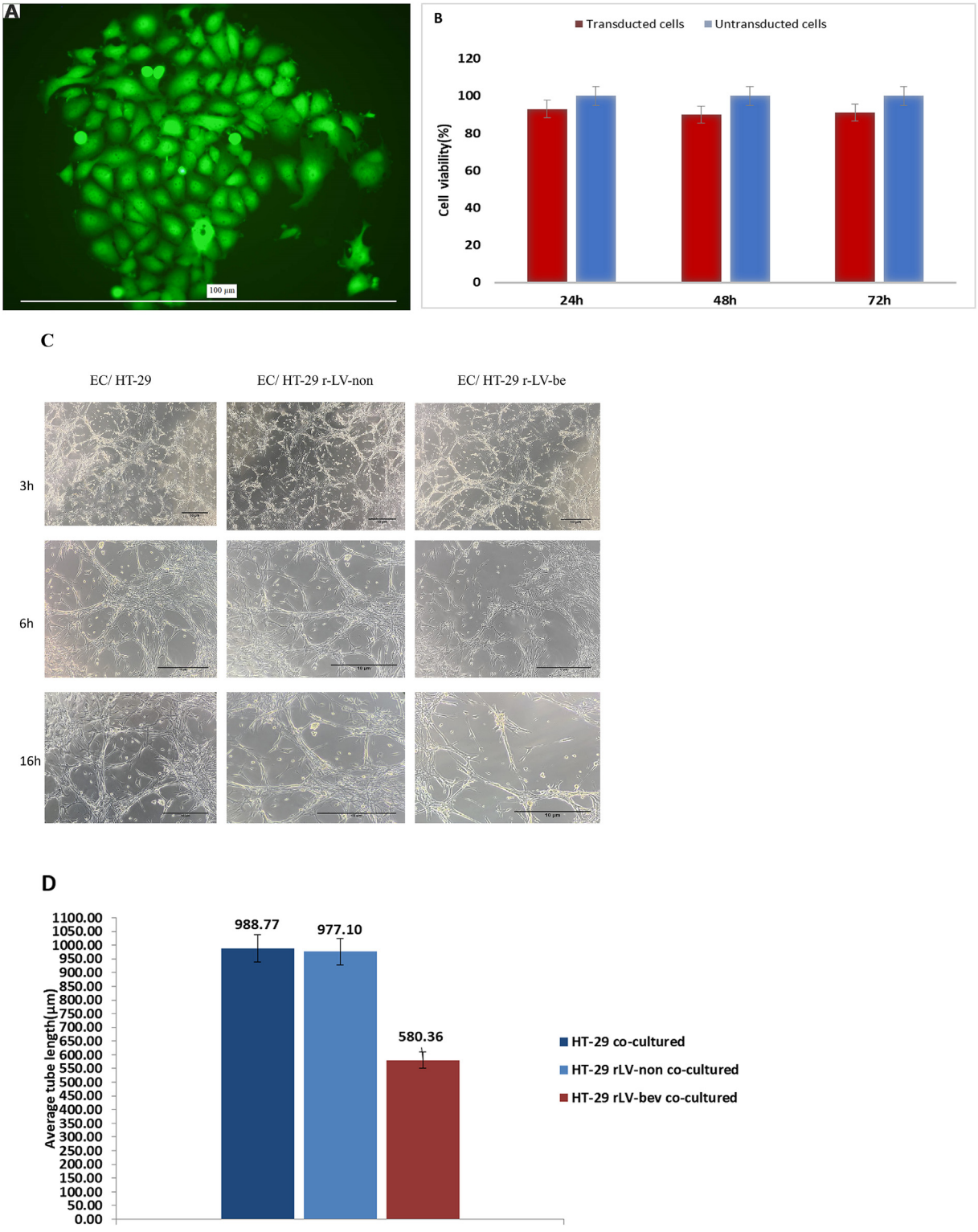

Fig. 3. Expression and functional analysis of recombinant bevacizumab in HT-29 cancer cells. A. Expression of copaGFP detected by fluorescent microscopy shows the expression of bevacizumab in HT-29 cells transduced with rLV-bev viral particles B. Cell viability of HT-29 cells transduced with rLV-bev or rLV-non viral particles. C. Tube formation assay in the co-culture of EC/HT-29 cells 24 h after seeding. EC/HT29-rLV-bev and EC/HT29-rLV-non are co-culture of EC with HT-29 cells transduced with rLV-bev and by rLV-non viral particles respectively. Tube formation was inspected in 3, 6, 16 h and the visual patterns were defined by photographing the cells under phase contrast microscope, 10 × –100 × magnification. D. Comparison of average tube length formation for the co-culture of EC/HT29, EC/HT29-rLV-non, and EC/HT29-rLV-bev.

active. The results of tube formation assay reveal that rLV-bev transduced cancer cells (HT-29) express functional bevacizumab mAb which significantly suppressed tube formation in a co-culture system included with rLV-bev transduced HT-29 cells and EC. While tube formation assay confirms the functionality of bevacizumab mAb produced by our designed lentiviral gene delivery system, the anti-angiogenesis and anti-cancer effects of the developed system needs further studies in animal models.

5. Conclusion

We report the development of a lentiviral vector expressing recombinant bevacizumab. Our results show that the designed gene delivery system efficiently produce full-length bevacizumab mAb in cancer cells. We also confirmed the bioactivity of recombinant bevacizumab produced by colon cancer cells transduced by rLV-bev lentiviral vectors. These findings suggest that the developed gene-based anti-VEGF mAb system has a potential for angiogenesis-targeted therapy of cancer. Further studies are suggested to assess the anti-angiogenesis and therapeutic efficacy of bevacizumab producing lentiviral system in animal cancer models *in vivo*.

Declaration of competing interest

The authors declare that they have no competing interests.

Acknowledgments

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