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An Engineered Mesenchymal Stem Cell by Lentiviral Vector Expressing CD44 as a Candidate to Target Colon Cancer Tissue in Mice Model

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ABSTRACT

Backgrounds: It is evident that the success of common cancer treatments is reduced due to limited drug access to tumor tissue, the drug toxicity intolerance in healthy cells, as well as the exposure of the immune system to the drug. Cancer stem cells are also a small population of tumor cells, which have different potentials for regeneration, proliferation, and differentiation and serve as a carcinogenic driving force. They are believed to play a key role in the onset, progression, drug resistance, recurrence of cancer, or metastasis. Although mesenchymal stem cells (MSC) have a slight ability to migrate toward the tumor, they could be considered as a cellular carrier for tumor targeting due to lack of recognition by the host immune system. Stem cells with their own ligands could effectively target cancer cells. One of the CD markers that exist on the surface of stem cells is CD44v6, which is considered as a homing receptor. Given that the expression level of stem cell markers is reduced during consecutive cultures in vitro environment; therefore, in the present study, stem cells were engineered using CD44 lentiviral vectors to more effectively improve the implantation and targeting of the colon cancer cell model.

Materials & Methods: In this study, the structure of the CD44 gene was designed in lentiviral vectors and transfected to the HEK293T cell line along with auxiliary plasmids PSPAX2 and PMDG2. The growth medium of virus-containing cells was collected at optimized intervals, and transduction into mice mesenchymal stem cells, injection into mice, and homing processes were traced.

Findings: Successful production of lentiviral vectors and proper expression of the corresponding factor after transduction were effective in improving the MSC homing in cancer cell.

Findings: According to these findings, it could be suggested that high expression of CD44v6 factor could be effective in improving the implantation process in cancer cells and targeting treatment.

Keywords: CD44, Mesenchymal stem cell, Homing factor.

CITATION LINKS

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Introduction

Developing strategies targeting cancer stem cells through drug carriers, specific surface markers, inhibition of signaling pathways or their components, and elimination of tumor micro-environment could improve the clinical outcomes of cancer patients [1].

In general, it could be noted that any approach that targets cancer cells, cancer stem cells, and stromal components of the tumor could lead to advances in cancer treatment [2-4].

Colorectal cancer is the development of cancer from the colon to the rectum, and the treatments used for colorectal cancer may include a combination of surgery, radiation therapy, chemotherapy, and targeted therapy. The survival rate of patient for the next five years is estimated to be 65%, and the likelihood of patient survival depends on the cancer progression. Unfortunately, the disease has become more common in recent years, so that traditional treatment strategies are no longer effective.

Many studies support the role of intrinsic tendency of stem cells toward malignant and invading tumors. Although molecular and targeted mechanisms are still under investigation, discovering the capability of stem cells in pursuing tumors is one of the new ways that could be employed to develop targeted therapies for metastatic malignancies. In recent years, several studies have shown that it is possible to transport stem cells by pairing them with various biological agents, including cell suicide genes. It seems variation in stem cell structure is well documented in previous studies; this particular characteristic makes stem cells more effective than all other carriers for use in drug/viral anticancer therapy. Different stem cells could be used as carrier cells. These cells could be extracted from a variety of tissue sources, including mesenchymal stem cells (MSCs), neuronal stem cells (NSCs), and fat-derived stem cells [5-7].

Mesenchymal stem cells with the ability to self-regenerate, differentiate into multiple cell types, transfect, modify Ex-vivo, and migrate toward tumors have been considered as attractive treatment options [8].

On normal cell surfaces, there are glycoproteins that play important roles in intercellular communication, cell-tissue communication, and evolution. These glycoproteins perform a wide range of functions in the cell; for example, some of them may be receivers, and some may be involved in binding to tissues. One of the CD markers on the cell surface is CD44, which is a strong homing receptor [5].

The CD44 gene is located on the short arm of chromosome 11 and consists of about 20 exons, of which 12 are involved in cellular junctions. CD44 also has more than 20 isoforms, and the functions of about 12 isoforms have been discovered [5, 9].

CD44 normally binds to its primary ligand, hyaluronic acid. This binding is thought to be responsible for cellular signaling and the regulation of other biological processes within cells. Cells within the tissue interact with each other either through the intracellular matrix or through cell junctions. CD44, as an adhesion molecule, enables cell communication by transmitting cell-to-cell signals. In addition to its role in cell adhesion, CD44 could conduct intracellular signals for growth and movement. Cells that are positive for the CD44 marker have also been shown to exhibit mesenchymal cell profiles [9].

It should be noted that some CD44 isoforms, including CD44v6, play a role in most cancers, including breast and colon. Binding of CD44 to hyaluronic acid is involved in the regulation of homing of stem cells and could also be effective in calling mesenchymal cells to the site of inflammation and damage [10].

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Recent evidence suggests a decrease in the expression of stem cell surface markers during frequent cultures in vitro. As a result, by increasing the expression of CD44, implantation in cancer stem cells could be improved to some extent [11].

Lentiviral vectors are one of the viral carriers that are considered for this purpose due to their salient properties such as long-term expression [12, 13].

Objectives: This study aimed to increase the expression of CD44 marker on the surface of stem cells and outline its effective role in tumor targeting and also highlight the prospects of targeted treatment. This research was approved by the Ethics Committee of Tarbiat Modares University on April 8, 2018 (52D/108).

Materials and Methods

Cloning of CD44v6 in PCDH: Based on previous investigation, the CD44v6 gene was prepared from the gene bank (Accession #: NM001177787.1), and the sequence was synthesized and inserted in pCDH-CMV-MCS-EF1 (addgene cat #:72263) by XbaI and KpnI (Thermo fisher USA) based on the procedures recommended by the manufacturer.

Confirmation of successful cloning was performed by enzymatic digestion using two enzymes, *XbaI* and *Kpn1*. In order to confirm the ligation, semi-nested primers were designed (one inside the CD44 gene fragment, the other primer in the PCDH plasmid, and a reverse primer inside the CD44 gene) and sent to Pishgam Company for synthesis (Table 1). The cloned plasmid as well as psPAX2 and pMD2.G, as packaging and helper plasmids, were transfected into HEK293T cell line by PEI (polyethylenimine) (Sigma, Germany). After 48 hours of incubation at 37 °C with 5% Co_2, the lentiviral vector was harvested (Figure 1 and 2).

Production of lentiviruses: In this study, 293T cells were cultivated and plated 24 hrs before transduction. Cells were cultured in DMEM (Biosera, France) with 10% fetal bovine serum. After 2 hrs of incubation in serum-free medium at 37 °C, the cells were washed, and the medium was changed. The cell supernatant containing lentiviruses was collected 24, 48, and 72 hrs post transfection and centrifuged at 1500 rpm for 10 min. To improve the attachment of the cells to the bottom of the plates, polybrene was also added based on the manufacturer's instructions (Sigma-Aldrich Company). Lentivirus particles were harvested and concentrated by ultra-centrifugation at 25000 g for 2 hours.

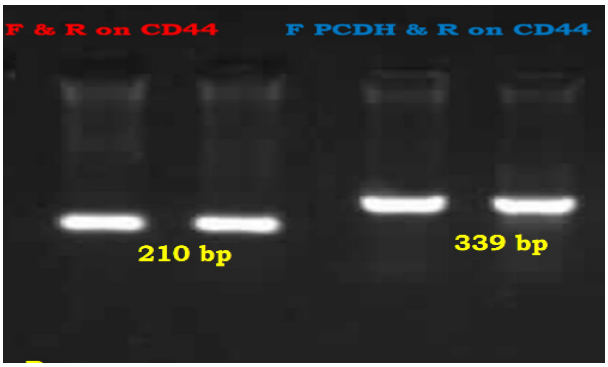

Figure 1) forward primer on PCDH plasmid and reverse primer on CD44v6 gene, which resulted in a 210 and 339 bp fragments

Adipose-derived mesenchymal stem cells from Balb/c mice: Obtaining a stem cell population requires several sequential steps, including harvesting, mechanical breakdown, chemical breakdown, purification, and adherence. Adipose tissue was taken from the abdomen by operation procedure in a sterile biological cabinet, and stem cells were harvested by chopping and using 1 mg/mL of collagenase I enzyme. The cells were treated with collagenase I (Gibco, USA) and cultivated in DMEM-F12 (Biosera, France) with 20% calf serum (Gibco, USA) and 1x pen strep (Biosera, France). The cells were incubated at

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37 °C with 5% CO_2 and inspected daily with inverted microscopy. After two passages, the homogeneous population of fibroblast-liked cells was observed and confirmed by immuno-phenotyping technique.

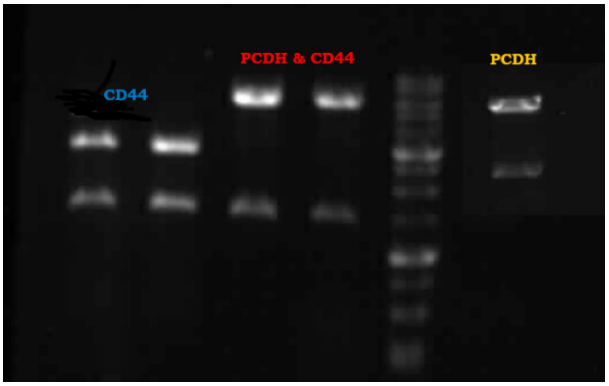

Figure 2) Enzymatic digestion of PCDH and CD44 by *XbaI* and *Kpn1* enzymes and ligation in PCDH plasmid.

Titration of Recombinant lentiviruses: Transforming units of lentiviruses (TU/mL) were determined by analyzing the number of virus particles able to transduce mesenchymal stem cells. To do so, cells were seeded on 6-well plates at 60000 cells per well. Lentivirus transduction with serial dilutions was carried out on the second day. On day 5, the cells were analyzed with flow cytometry technique to reveal the percentage of cells which were transduced by CD44v6-expressing lentiviruses based on a specific antibody.

Vector titers were calculated using the following formula:

TU/mL= (Number of cells transduced x percent fluorescent)/ (virus volume in mL)

Evaluation of homing of engineered mesenchymal stem cells containing CD44v6 in Balb/c mice: Following the growth and expansion of CT26 mouse colon carcinoma cell line *in vitro*, trypsinized cells were harvested, washed, and counted by trypan blue dye exclusion method. A suspension of 6×10^5 viable tumor cells in 0.2 mL of PBS (phosphate-buffered saline) was inoculated into the right flanks of Balb/C mice. After the tumors developed to a volume of about 80-100±10 mm^3, the mice were euthanized, and 6 x 10^5 engineered stem cells (carrying CD44v6 gene) were injected subcutaneously into adjacent or opposite flanks of the tumor in mice. In order to trace homing, the GFP reporter gene was also transfected into stem cells for the sedation in mice. Compounds such as ketamine and xylazine were used as the drug of choice for anesthesia in mice as suggested, and the study groups were transferred to the Preclinical Center of Tehran University 24 hours after injection. After absorbing the fluorescent material in the desired position, the laser light was simulated by the KODAK in-vivo FX multispectral image system.

Five mice were included in each group based on the below pattern:

Group 1: Administered by injection of MSCs carrying Lenti-CD44v6 vector adjacent to the tumor

Group 2: Administered by injection of MSCs carrying Lenti-CD44v6 vector on the opposite flank of the tumor.

Group 3: Negative control without injection in tumor mice model.

Findings

Confirmation of ligation process: The

Table 1) Semi-nested primers to check CD44v6 gene and PCDH plasmid

	Primer	Sequence 5'.....3'	Length
1	Forward CD44	5'CACACAGCTTGGGGACTTTG3'	20
2	Reverse CD44	5'CTTGCTCAGGGCCAACTT3'	18
3	Forward PCDH	5'AATGGGCGGTAGGCGTGTA3'	19

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accuracy of cloning of the desired fragment in PCDH vector was confirmed with semi-nested PCR as well as specific primers for PCDH. The reaction was optimized based on chess board rules, and the optimized technique was used.

Confirmation of the ligation process by enzymatic digestion: To finalize the ligation process, enzymatic digestion was performed by XbaI and Kpn1 enzymes on colonies grown on plate. A 1407-bp fragment of the CD44 gene was isolated from the PCDH vector.

Therefore, after electrophoresis of the plasmids digested on the gel, a 1407 bp band corresponding to the CD44 fragment and a 5583 bp band corresponding to the PCDH vector were visible in the figure. Plasmid was extracted, and enzymatic digestion was performed using two enzymes Kpn1 and XbaI.

Transfection of structures containing the CD44 gene into HEK293T cells: Transfection of the designed structures was performed using PEI, and RNA extraction and cDNA synthesis were performed on the same day. The result of this transfection could be evaluated by performing real-time PCR test. The CD44 gene amplification and melting curve analysis were done as shown below.

The DNA amount in a CD44 gene among non-treated samples was estimated using the $2^{-\Delta\Delta CT}$ method.

The results showed that CD44 gene expression increased approximately 4-fold within 48 and 72 hours after transfection compared to the control.

Transfection of HEK293 cells was performed with PCDH-CD44, psPAX2, and pMD2G vectors using PEI. Cytopathic effects (change in cell morphology and formation of large clusters compared to normal cells) were observed after 48 hours using an inverted microscope.

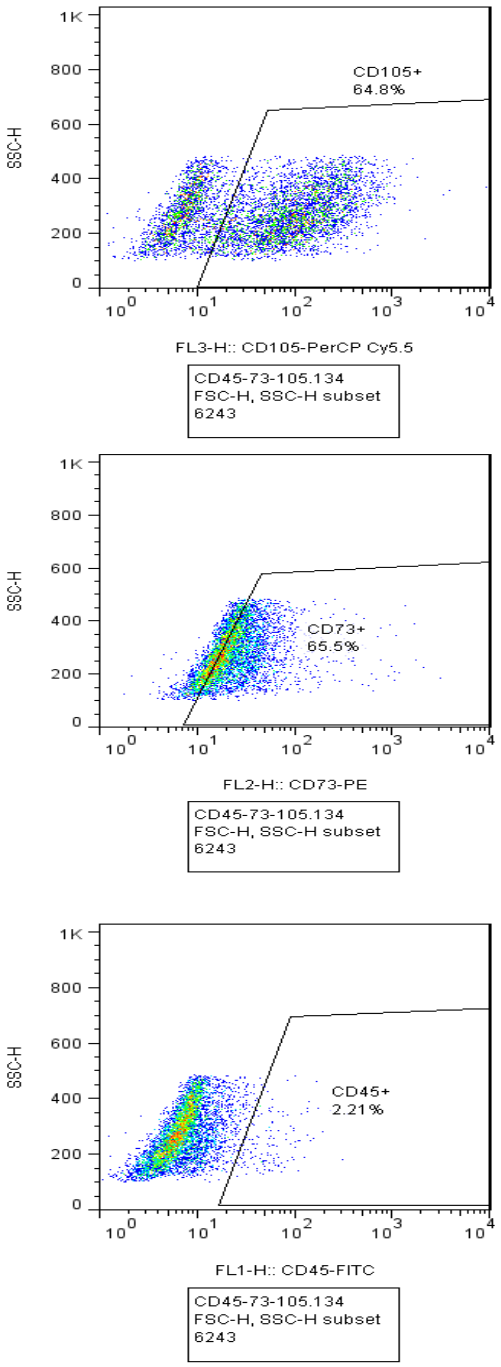

Figure 3) Evaluation of mesenchymal stem cell markers by flow cytometry. Adipose tissue cultures with CD105, CD73 and CD45 immuno-phenotype at 2nd passages. Top right shows the percentage of CD105, CD73, and CD45 markers on mesenchymal stem cells from Balb/c mice. The expression levels of CD105 and CD73 were 64.8 and 65.5%, respectively. CD45 as negative marker was only about 2.21%. The absorption and diffusion wavelength of Percp-cy5.5, PE, and FITC markers were adjusted to 482 and 676, 496 and 578, as well as 494 and 520 nm, respectively.

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Immuno-typing: Expanded clonal cells were stained with CD105, CD73, and CD45 monoclonal antibodies. CD105 and CD73 were selected as positive phenotype markers and cd45 as a negative marker (Figure 3).

Therapeutic CD44v6 transgene expression analysis by flow cytometry: CD44v6 expression was evaluated as a marker in mesenchymal stem cells using mouse CD44v6 monoclonal antibody by flow cytometry. Then the percentage of transduced mice MSCs was determined by flow cytometry as $5.1x10^8$ TU/mL (Figure 4).

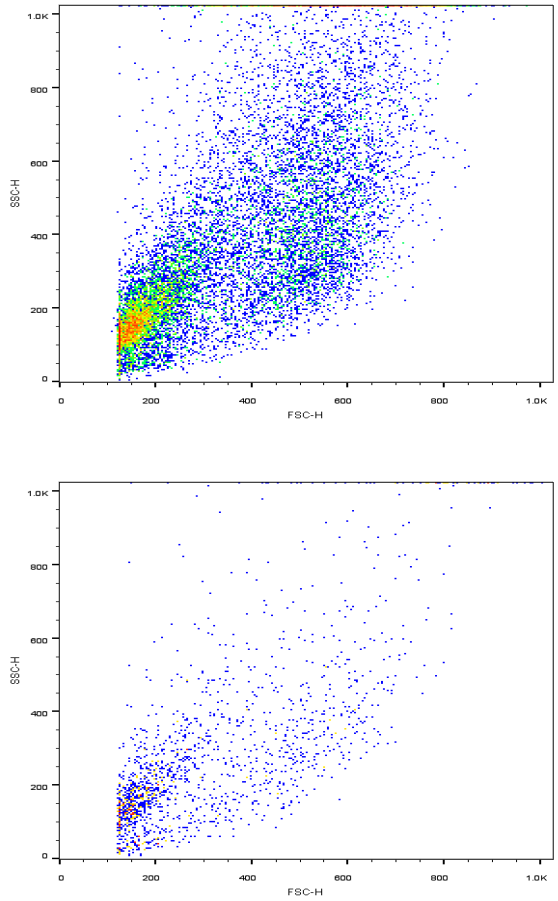

Figure 4) Transduction of CD44v6 to mesenchymal cells by lentiviral vector.
Transduction efficiency was measured and evaluated by comparing the expression of CD44v6 as well as controls and it was about 82%.

In vivo study in mice model: Pre-clinically, the murine CT26 colon cell line is considered as a suitable platform model for evaluating and studying colon cancer microenvironment and homing. The male model of four to six-week-old Balb/C mice was used to create a tumor model. The average time of tumor formation in these mice was five days.

In vivo detection of homing capacity of engineered mesenchymal stem cells expressing CD44v6 in Balb/c mice: In vivo imaging was performed using Kodak imaging system (FX pro system) with fluorescent. Exposure time was 60 seconds, and excitation and emission filters were set to 470 and 535 nm, respectively (Figure 5).

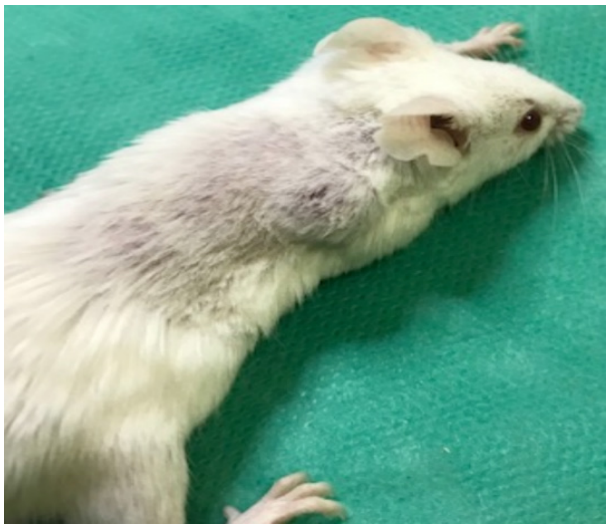

Figure 5) The tumor model created is visible on the right side of the mouse

The scan results in mice groups showed that the more the movement from the colors with lower wavelengths to the colors with higher wavelengths, the more the fluorescent amount. Also, when mesenchymal stem cells were used with more expression of the CD44v6 marker, the implantation process was induced in a more targeted manner.
Mesenchymal stem cells containing lentiviruses expressing the CD44v6 gene showed higher homing capacity in colon cancer in Balb/c mice (Figure 6).

Discussion

One of the main problems in cancer treatment

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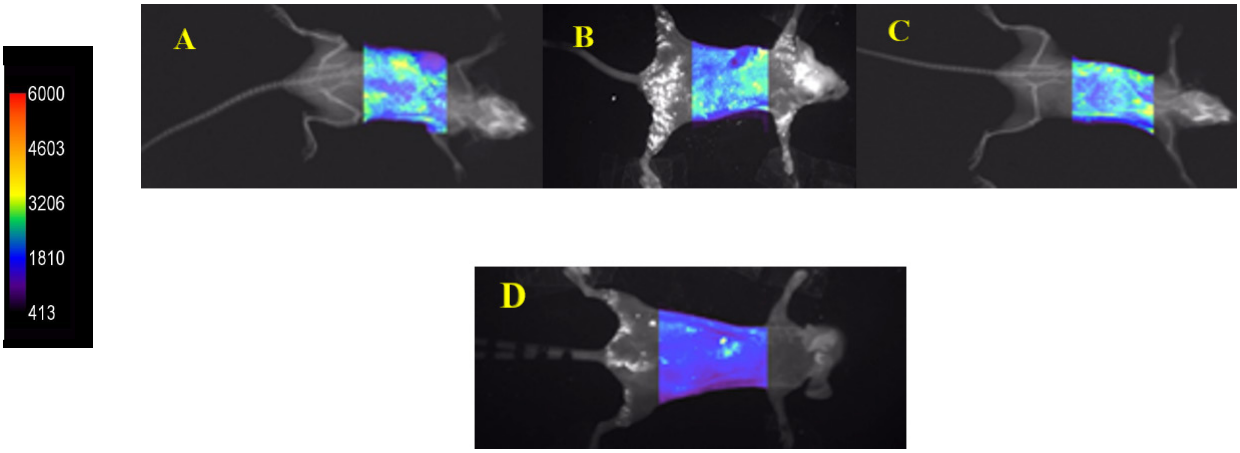

Figure 6) Results of the implantation process at the tumor site by in vivo scanning technique in Bab/c mice with subcutaneous Kodak imaging system. Figure A: a group that systemically received recombinant lentivirus expressing CD44 introduced into mesenchymal stem cells. Image B: a group of mice administered by injection of recombinant lentivirus expressing CD44 along with mesenchymal stem cell around the tumor. Image C: a group of mice administered by systemic injection of mesenchymal stem cell. Image D: tumor-free mice without injection. The results show that signal change in animals that received the cd44 marker was induced 10 hours after injection in the implantation control group. The control group had a tumor lesion on the right side, but did not receive the desired marker; thus, no noticeable change was induced in the tumor area.

is related to targeting the tumor tissue and thus the targeted transfer of therapeutic agents such as drugs or oncolytic viruses to the tumor site. Studies have shown that stem cells have the ability to migrate to tumors and wounds and could be used as carriers of therapeutic agents to the tumor site [14].

Because of the systemic nature of many diseases and the tendency to non-invasive treatment, systemic mesenchymal stem cell injection, which results in tissue regeneration and immunosuppressive effects, is an extraordinary therapeutic perspective [15, 16]. The significance of these results is that the systemic delivery of mesenchymal stem cells to the tumor site without specific targeting may affect their potential as a therapeutic agent [5].

Among stem cell surface markers, CD44 surface antigen is considered as a homing factor. This surface antigen is essential for cell-to-cell interaction between germ cells and their surrounding cells and preserves stem cell integrity. In addition, CD44 is an important marker in implantation and migration of precursor stem cells. On the other hand, the interaction of CD44 with hyaluronic acid leads to skeletal changes that enhance the stimulation and attack on tumor cells. Most of the available evidence suggests that various types of CD44 are associated with these interactions [5, 17].

It is believed that increasing the expression of CD44 marker on the stem cell surface is more efficient in the homing process; thus, in this study, a treatment was compared to the control to evaluate the hypothesis. Studies were begun based on the strength points of promising studies. The focus of the present study was to show how to use mesenchymal stem cells to target cancer cells and optimize drug delivery with minimal cytotoxicity effects.

Conclusion

This study showed that the reduction of CD44 marker expression in culture medium was partially improved by transfection of the construct containing the relevant gene, which could be effective in the process of implantation into cancer cells and targeted treatment.

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Recent evidence suggests that engineered stem cells could play a unique role in targeting tumors by allowing anti-cancer gene products to be delivered directly to local and invasive cancer foci. Based on the current research results, it is concluded that since increased CD44v6 expression increases the potential of cell homing, engineered mesenchymal stem cells with increased CD44v6 expression on the cell surface could dramatically show higher homing capacity and may be used for further investigation and targeted therapy in colorectal cancer.

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Ethical Permissions: The research was approved by the ethical comity of Tarbiat Modares University. (IR.TMU.REC.1396.715).

Conflicts of interest: There is no conflict of Interest to declare.

Authors Contribution: Conceptualization: MR, AN; data curation: AN; formal analysis: AN, MR; funding acquisition: MR; investigation; MR methodology: MR; project administration: MR; software: AN; supervision: MR; writing of the original draft: AN; writing-review and editing: MR.

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Consent to participate: Not applicable.

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