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A novel reconstruction and expression of endostatin with pDC316 plasmid in eukaryotic cells

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Abstract

Endostatin is one of the most potent endothelial cell inhibitory factors that has been discovered so far, however, the endostatin protein is extremely unstable and difficult to manufacture and store. The objective of this research was to investigate a novel biotechnology method to manufacture endostatin. This was accomplished by subcloning the endostatin gene into a pDC316 plasmid and transfecting the desired transformed plasmid into human embryonic kidney 293T cells. The Endostatin gene DNA was amplified by the polymerase chain reaction (PCR). Purified endostatin gene fragments were ligated to a eukaryotic expression vector, pDC316. The reconstructed plasmids were transformed into competent *E. coli* strain DH5α cells in order to amplify and facilitate the extraction of desired high-purity plasmids. Single and clear-edged bacteria were tested by bacterial PCR and electrophoresis. The desired plasmid was extracted from the transformed bacteria and was sequenced for further confirmation. The endostatin containing plasmid was subsequently transfected into 293T cells, the expression of the target gene in the transformed cells was detected by reverse transcription PCR (RT-PCR) and by gel electrophoresis. This recombinant endostatin has potential value in angiogenesis gene therapy.

Keywords

Recombinant endostatin, Angiogenesis, pDC316, 293T cells

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Introduction

More than 40 years ago, Judah Folkman published a revolutionary new way to think about cancer and shared his new hypothesis that tumor growth is angiogenesis dependent [1]. Angiogenesis is the physiological process by which new blood vessels form from pre-existing blood vessels [2]. Over the years, a large body of evidence was found to support Folkman's idea and research on angiogenesis blossomed [1]. In 1994, O'Reilly et al. discovered the first anti-angiogenic peptide named angiostatin, and since the discovery of angiostatin, other anti-angiogenic peptides have been discovered [3]. Endostatin was first found in mouse cells in 1997 also by O'Reilly's group and was subsequently found in humans [4]. The discovery of endostatin was a breakthrough that led to a thorough understanding of angiogenesis [3].

Endostatin is a 20-kDa protein fragment located at the C-terminal of the noncollagenous (NC1) domain of the type XVIII collagen, where it occurs as a globular protein in humans [3,5]. Under normal physiological circumstances, angiogenesis is tightly controlled by a variety of chemical signals in the body; one of them being the vascular endothelial growth factor (VEGF) [6]. Endostatin can specifically inhibit the proliferation of vascular endothelial cells treated with basic fibroblast growth factor (bFGF) and induce endothelial cell apoptosis [4].

Uncontrolled angiogenesis leads to pathological hyperplasia and neoplastic disease [7,8]. It has been established that tumor growth and carcinogenesis depend on angiogenesis [9]. A variety of experiments have confirmed that endostatin has an inhibitory effect on growing blood vessels, but has no effect on resting blood vessels; a selective effect by which it can effectively limit uncontrolled angiogenesis [1]. The inhibitory effect of endostatin on angiogenesis is very important and underscores its potential as a cancer treating agent.

In recent years, research that focuses on endostatin anti-tumor angiogenesis therapy has intensified. The production of recombinant endostatin is difficult and yield is low, therefore, different expression systems have been used in different studies. They include expression in: *E. coli*, yeast, and in mammalian cells [10,11]. The *E. coli* expression system has a high yield, but the products mostly exist in the form of insoluble inclusion bodies, necessitating further downstream processing and purification [12]. Although the protein is soluble after refolding, a large amount of protein is lost during this process. The expression product of mammalian cells has high biological activity, but low expression yield and difficult to purify [13]. Although the yeast expression system has a high yield and is easy to purify [14], the transduction of the endostatin gene through an appropriate vector is a more suitable method because of its higher yield and efficiency. The endostatin gene is delivered by an appropriate vector to provide for long-term and stable expression in the body. It has been confirmed that liposomes, adenovirus, retrovirus, adeno-associated virus, and lentivirus are all effective vectors for delivering the endostatin gene [15]. Compared with non-viral vectors, higher plasma concentrations of endostatin are generally obtained after transfection with viral vectors.

In this study, endostatin was reconstructed by using a pDC316 plasmid, which was then transfected into human embryonic kidney 293T cells. RT-PCR and electrophoresis were used to confirm the production of endostatin RNA in 293T cells.

Materials

The deoxynucleoside triphosphate (dNTP), *Thermococcus kodakaraensis* (KOD) buffer,

KOD enzyme, type II restriction enzymes, CutSmart® buffer, t4 DNA ligase, t4 buffer were obtained from New England Biolabs, Ipswich, MA. The endostatin cDNA template, Forward (F) and Reverse (R) primers were obtained from Sangon Guangzhou, Huangpu District, China. The Taq PCR master mix was obtained from Tiangen Biotech Co., Ltd, Haidian district, Beijing, China. The pDC316 plasmid was obtained from Youbio, Yuhua district, Changsha, Hunan province, China. The bacto-tryptone, yeast extract, and agar were obtained from Guangdong Huankai microbial Sci&Tech Co. Ltd, Guangzhou Development District, Huangpu, Guangzhou province, China. Ampicillin was obtained from Guangzhou saiguo biotech Co. Ltd, Tianhe district, Guangzhou province, China. SuperRT® one-step buffer and SuperRT® one-step Enzyme Mix, RNA extraction kit and the SuperRT® one step RT-PCR kit were obtained from Jiangsu Cowin Biotech Co. Ltd, Wuhan, Hubei, China. The PEI transfection protocol and kit was obtained from Polysciences, Inc., Warrington, PA.

The *E. coli* DH5α strain was obtained from Hunan Fenghui Biotechnology Co. Ltd, Yuelu district, Changsha, Hunan province, China. The 293T cells were obtained from the Sun Yat-sen University cancer center, Guangzhou, China.

Electrophoresis was performed using a EPS-300, Tanon Science & Technology Co. Ltd, MinXing District, Shanghai, China. The lab scale shaker model no. ZHWY-100D was manufactured by ZhiCheng analytical instruments manufacturing Co. Ltd, Fenshan District, Shanghai, China. The E.Z.N.A® gel extraction kit was obtained from Omega Bio-tek, Inc., Norcross, GA. Where the procedure for a unit operation is not presented, it was performed per the manufacturer's instructions. The pDC316 plasmid was annotated with SnapGene®, GSL Biotech LLC, San Diego, CA, http://snapgene.com.

Methods

Combining the Endostatin fragment with the pDC316 vector

The Endostatin segment was amplified by using polymerase chain reaction. The 50 ul reaction system was composed of: water 33.7 ul, dNTP 5 ul, 5x KOD buffer 5 ul , both F and R primer 1.5 ul , endostatin cDNA template 0.3 ul , KOD enzyme 1ul , and $MgSO_4$ solvent 2 ul. The sequence of the F and R primers is presented in Table 1.

The PCR conditions were designed as follows: initial denaturation at 94°C for 2 minutes, 35 cycles of 98°C for 10 seconds each, 60°C for 30 seconds and 68°C for 55 seconds followed by final extension at 68°C for 2 min (Table 2).

Table 1. Primer information

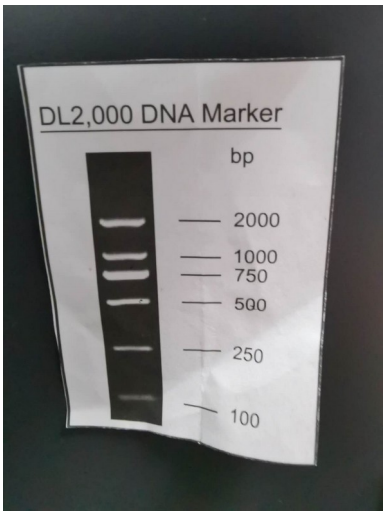
Name	Sequence	number of bases	OD /tube	Nmol/ tube	water added (μl)
he10a_F_B1	5'CGGGATCCATGGCCCACAGCCACCGGG3'	27	2	7.99	79.9
he10A_R_H3	5'CCCAAGCTTTCACTTGGAGGCTGTCATAAAGC3'	32	2	6.74	67.4

Table 2. PCR conditions to amplify the endostatin segment

Step	1	2	3	4	5
Temperature (°C)	94	98	60	68	68
Time (min:sec)	2:00	0:10	0:30	0:55	2:00
Number of cycles	0	35	0	0	0

After PCR was completed, the sample were electrophoresed by adding the DNA loading buffer with the ratio of 4:1. A marker (1x) for comparison was also added in the gel (Figure 1). The gel was allowed to run for 25 min at 125 V. The same marker and gel setting was used for all gel electrophoresis experiments in the study.

Figure 1. The marker

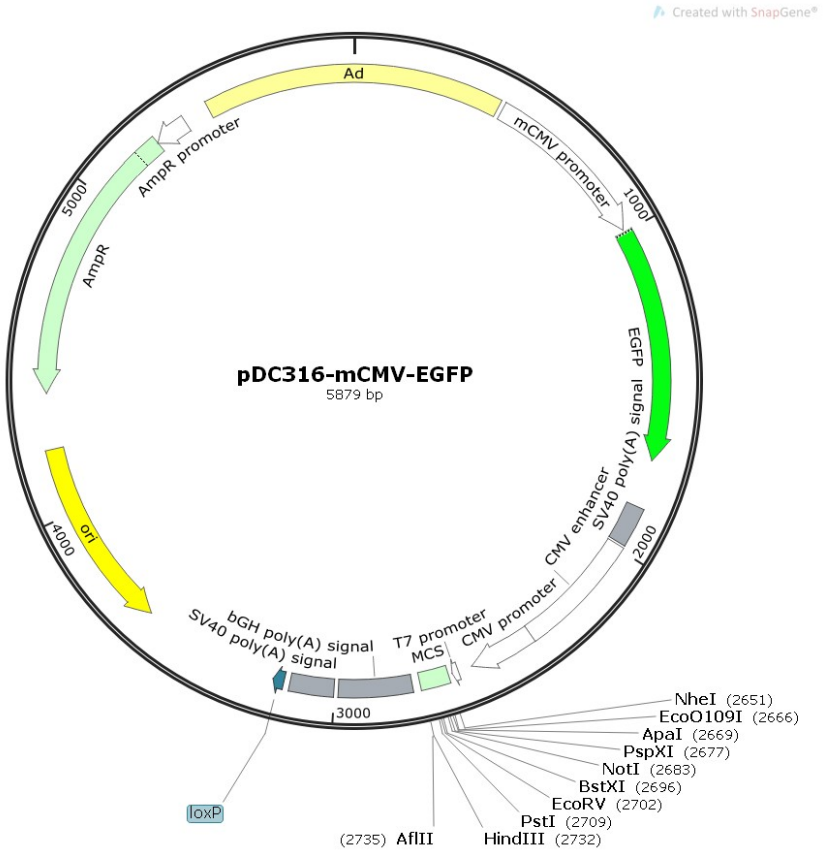


After the electrophoresis was completed, gel extraction was performed by using the E.Z.N.A^{R} Gel Extraction Kit in order to obtain a pure, single PCR product and gene ladder. Next, Hind III and BamH I which are two type II restriction enzymes were used to digest the results from the gel extraction. The 20 ul reaction system was composed of the plasmid pDC316 vector and cDNA templates 10 ul, BamH I enzyme 1 ul, Hind III enzyme 1 ul, CutSmart® buffer 2 ul, and water 6 ul. In the

process of enzyme digestion, the tube was immersed in a 37 °C water bath for 3 hours.

Those DNA segments were ligated to the pDC316 plasmid (Figure 2) by adding t4 DNA ligase. The 20 ul reaction system was composed of water 15.3 ul, DNA segments 1.4 ul, pDC316 plasmid 0.3 ul, t4 DNA ligase 1ul, and t4 buffer 2 ul. The mixture was incubated at room temperature for 3 hours.

Figure 2: pDC316 plasmid



Extraction of the identified plasmid

The products produced by ligation were transformed into competent *E. coli* DH5α cells with the volume 10 ul and 20 ul. During transformation, the ligation products were iced for 30 minutes, then placed in a 42°C water bath for 60 seconds, and then placed in ice again for 5 minutes. The resulting mixture was transferred evenly onto a LB petri dish, in a 100 ml scale composed of 1g bacto-tryptone, 0.5g yeast extract, 1g NaCl, q.s water to 100 ml, 0.5 g agar technical, 100 ul ampicillin solution with a concentration of 10 mg/mL. The petri dish was incubated in a 37°C incubator for 16 hours.

Bacteria that were single and clear-edged were selected from the petri dish after incubation and amplified by PCR. The 20 ul reaction system was composed of: water 5 ul, Taq PCR master mix 10 ul, both F and R primer 1ul, and bacteria template (made by 10 ul water and a single bacterial colony sample of 3ul. The bacterial PCR conditions were designed as follows: Initial denaturation at 95°C for 3

minutes, 30 cycles of 94°C for 25 sec, 60°C for 25 sec and 72°C for 55 sec followed by final extension at 72°C for 3 min (Table 3).

Table 3. Bacterial PCR conditions

Step	1	2	3	4	5
Temperature(°C)	95	94	60	72	72
Time(min:sec)	3:00	0:25	0:25	0:55	3:00
Number of cycles	0	30	0	0	0

After PCR amplification of bacterial colonies, the sample was electrophoresed for checking if the sample contained the same band as that from the first gel electrophoreses. This would indicate that the bacteria contained the identified plasmid. A single and clear-edged bacterial sample was selected and added into a 6 ml liquid nutrition medium (In a 100 ml scale composed of 1g bacto-tryptone, 0.5 g yeast extract, 1g NaCl, q.s with water to 100 ml, containing 100 ul ampicillin at a concentration of 10 mg/mL. The tube was placed on a laboratory shaker and shaken for 16 hours at a reciprocating speed of 200-220 rpm/min. The plasmids were collected by using the E.Z.N.A® endo-free plasmid extraction mini kit. The DNA sample was shipped at room temperature to Sangon Guangzhou for DNA sequencing.

Detecting the expression

Plasmids were transfected into 293T cells by following the PEI transfection protocol. RNA was extracted from the cells by following the instructions on the RNA extraction kit. The expression of the target gene in the transformed cells was detected by RT-PCR and electrophoresis. The 25 ul reaction system was composed of water 7.5 ul, SuperRT® onestep buffer 12.5 ul, SuperRT® onestep Enzyme Mix 0.5 ul, both F and R primers 1 ul,and RNA extracted from the cells 2.5 ul. The RT-PCR conditions were designed as follows: Initial denaturation at 95°C for 2 minutes, 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec followed by final extension at 72°C for 5 min (Table 4).

Table 4. RT-PCR conditions for the RNA extracted from 293T cells

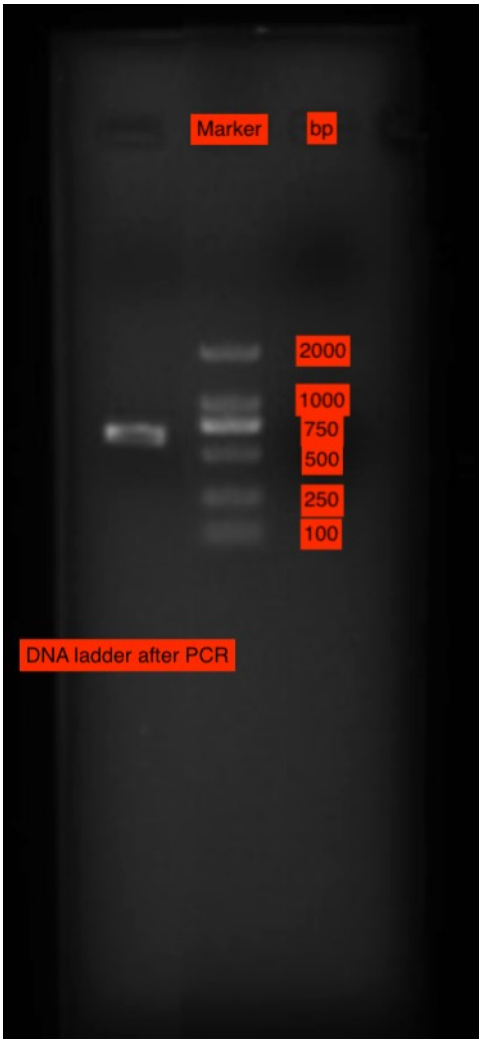
Step	1	2	3	4	5
Temperature(°C)	95	94	55	72	72
Time(min:sec)	2:00	0:30	0:30	0:30	5:00
Number of cycles		35			

After the RT-PCR procedure, the protein was identified using Agarose gel electrophoresis. 10 ul of the processed RNA sample was sent to Sangon Guangzhou for DNA sequencing and for confirming the result.

Results

Figure 3 shows the DNA band that was obtained after the first gel electrophoresis. The DNA band of endostatin is shown on the left and that of the marker is shown on the right. By comparing the DNA band of endostatin to the bands of the marker ladder, the DNA band of endostatin appears at > 500 bp, which matches the gene sequence of endostatin with 555 bp.

Figure 3. Electrophoresed DNA band of endostatin (left) and those of the marker (right) after PCR

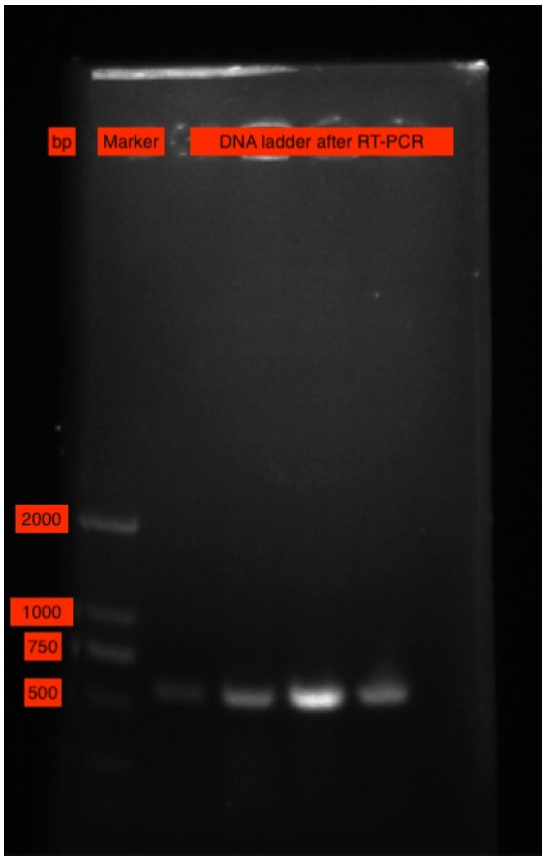


The DNA sequence of the extracted bacterial plasmid was sequenced by Sangon Guangzhou and was compared to the corresponding gene sequences using the Snapgene® software. The representative gene sequence is shown in Figure 5. No differences were found hence confirming that the extracted bacterial plasmid contained and expressed the endostatin gene.

The RNA produced by transfected 293T cells was reverse translated by the RT-PCR process using the SuperRT® one step RT-PCR kit. Figure 4 shows the DNA band that was obtained after RT-PCR. The first column shows the ladder of the marker, the other lanes being the band of the product DNA (four

samples were run in order to confirm the results). They were all approximately 500 bp, identical to the result of the first gel electrophoresis. Therefore, the transfected 293T cells were successfully shown to have expressed endostatin.

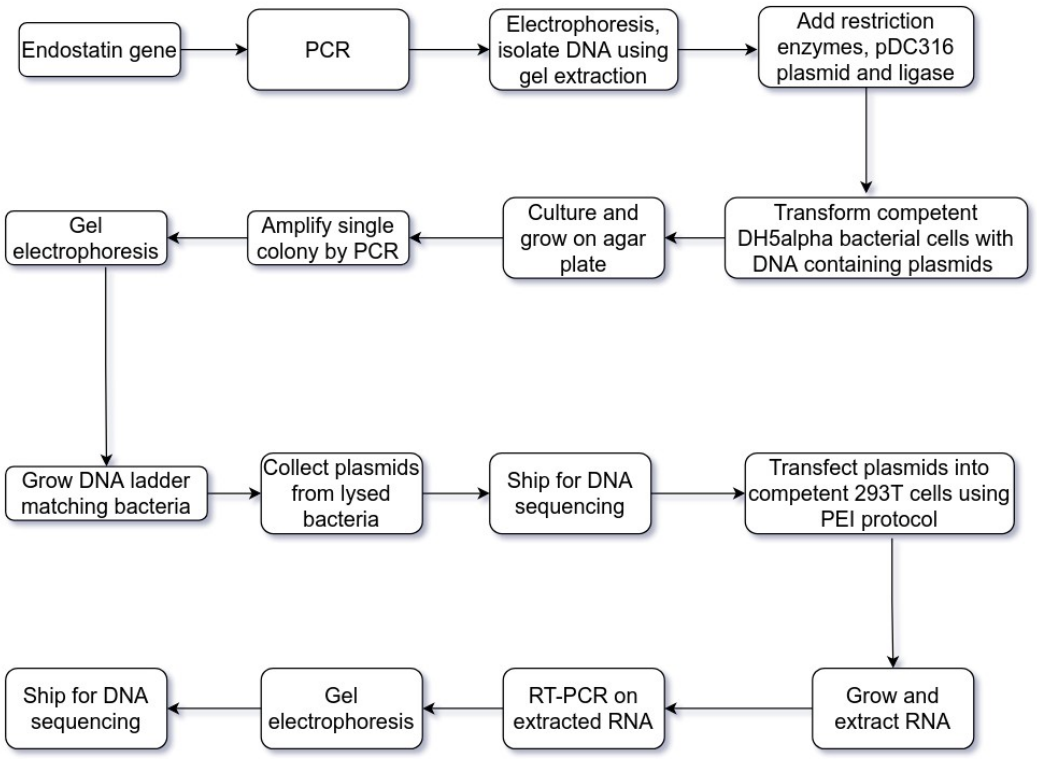
Figure 4. DNA ladder showed in the gel electrophoresis after RT-PCR.



The DNA sequence of reverse translated RNA from transfected 293T cells was sequenced by Sangon Guangzhou and was compared to the corresponding correct gene sequences using the Snapgene® software. There was no significant difference in the gene sequences. The representative gene sequence is shown in Figure 5. Therefore, the RNA produced by the 293T cells originated from the endostatin gene. The reconstruction of endostatin was hence deemed successful. A flowchart of the entire procedure is shown in Figure 6.

Figure 5: Gene sequence of Endostatin

Figure 6: A flowchart of the entire procedure



Discussion

pDC316 is an E1 shuttle plasmid, derived from the left end of the adenovirus type 5 (AD5) genome, which contains the human cytomegalovirus immediate early promoter, a polycloning site, and simian virus 40 (SV40) polyadenylation signals [16]. The plasmid pDC316 is very versatile, with both eukaryotic and prokaryotic shuttle capability and cell promoter originals. Also, pDC316 has two antibiotics genes which makes it easy for expansion. Last but not the least, pDC316 has a large capacity and can carry large gene fragments. Using the advantages of pDC316, this study successfully reconstructed endostatin into the pDC316 plasmid vector and transfected the identified plasmid into 293T cells, which subsequently expressed endostatin mRNA.

Several improvements can be made in this study. Subsequent to RT-PCR, the protein expression in transformed cells should be further confirmed by Western blotting. Western blotting is a useful technique that combines high efficiency gel electrophoresis and immunochemical analysis. Western blotting has the advantages of high sensitivity, large analysis capacity, and high efficiency. Western blotting is one of the most commonly used methods for detecting protein expression and distribution [17].

For future experiments, Adenovirus type 5 (AD5) can be used to package the reconstructed plasmid. Adenovirus is currently one of the most stable, reliable and efficient recombinant virus. It has the following advantages: 1. Wide host range. Adenovirus can infect a range of mammalian cells and it is highly compatible with 293 cells including 293T cells. 2. Because the infection of adenovirus does not depend on the cell cycle, it can infect both proliferating cells and non-proliferating cells. 3. Adenovirus has the characteristics of epithelial cells, because most tumor cells are epithelial cells, which makes adenovirus very suitable for the field of gene therapy. 4. It is strongly immunogenic [18,19]. After packaging the recombinant endostatin with adenovirus, it can be used to infect certain cells from the human body, such as vascular endothelial cells.

Since the pDC316 plasmid is derived from the AD5 genome, it is anticipated that the AD5 adenovirus will provide nearly ideal conditions for packaging the plasmid containing the recombinant endostatin gene. The AD5 adenovirus can potentially be used as a gene therapy for endostatin delivery.

Conclusion

Recombinant endostatin possesses significant inhibitory activity against a wide range of murine tumors. The human form of endostatin has also been produced in a recombinant form and exhibits antiangiogenic activity [20], which proves endostatin's potential value in future clinical applications. Several drugs related to recombinant endostatin are in the pipeline at different stages of clinical trials. It can be expected that more antiangiogenic drugs related to recombinant endostatin will become available for patients in the near future [21].

In conclusion, this study successfully reconstructed endostatin into a pDC316 plasmid vector and transfected the identified plasmid into 293T cells, which subsequently expressed endostatin mRNA. The final product is anticipated to have potential value in clinical trials and in angiogenesis gene therapy.

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References

1. Ribatti D. (2008). Judah Folkman, a pioneer in the study of angiogenesis. *Angiogenesis*, *11*(1), 3–10. https://doi.org/10.1007/s10456-008-9092-6

2. Wikipedia contributors. (2020, July 4). Angiogenesis. In *Wikipedia, The Free Encyclopedia*. Retrieved 14:01, September 4, 2020, from https://en.wikipedia.org/w/index.php?title=Angiogenesis&oldid=965958178

3. Walia, A., Yang, J. F., Huang, Y. H., Rosenblatt, M. I., Chang, J. H., & Azar, D. T. (2015). Endostatin's emerging roles in angiogenesis, lymphangiogenesis, disease, and clinical applications. *Biochimica et biophysica acta*, *1850*(12), 2422–2438. https://doi.org/10.1016/j.bbagen.2015.09.007

4. Wikipedia contributors. (2020, April 15). Endostatin. In *Wikipedia, The Free Encyclopedia*. Retrieved 13:26, September 4, 2020, from https://en.wikipedia.org/w/index.php?title=Endostatin&oldid=951078398
5. Javaherian, K., Lee, T. Y., Tjin Tham Sjin, R. M., Parris, G. E., & Hlatky, L. (2011). Two Endogenous Antiangiogenic Inhibitors, Endostatin and Angiostatin, Demonstrate Biphasic Curves in their Antitumor Profiles. *Dose-response : a publication of International Hormesis Society*, *9*(3), 369–376. https://doi.org/10.2203/dose-response.10-020.Javaherian

6. Rajabi, M., & Mousa, S. A. (2017). The Role of Angiogenesis in Cancer Treatment. *Biomedicines*, *5*(2), 34. https://doi.org/10.3390/biomedicines5020034

7. Yoo, S. Y., & Kwon, S. M. (2013). Angiogenesis and its therapeutic opportunities. *Mediators of inflammation*, *2013*, 127170. https://doi.org/10.1155/2013/127170

8. Gupta, K., & Zhang, J. (2005). Angiogenesis: a curse or cure?. *Postgraduate medical journal*, *81*(954), 236–242. https://doi.org/10.1136/pgmj.2004.023309

9. Nishida, N., Yano, H., Nishida, T., Kamura, T., & Kojiro, M. (2006). Angiogenesis in cancer. *Vascular health and risk management*, *2*(3), 213–219. https://doi.org/10.2147/vhrm.2006.2.3.213

10. Mohajeri, A., Sanaei, S., Kiafar, F., Fattahi, A., Khalili, M., & Zarghami, N. (2017). The Challenges of Recombinant Endostatin in Clinical Application: Focus on the Different Expression Systems and Molecular Bioengineering. *Advanced pharmaceutical bulletin*, *7*(1), 21–34. https://doi.org/10.15171/apb.2017.004

11. Mohajeri, A., Pilehvar-Soltanahmadi, Y., Pourhassan-Moghaddam, M., Abdolalizadeh, J., Karimi, P., & Zarghami, N. (2016). Cloning and Expression of Recombinant Human Endostatin in Periplasm of Escherichia coli Expression System. *Advanced pharmaceutical bulletin*, *6*(2), 187–194. https://doi.org/10.15171/apb.2016.026

12. Singh, A., Upadhyay, V., Upadhyay, A. K., Singh, S. M., & Panda, A. K. (2015). Protein recovery from inclusion bodies of Escherichia coli using mild solubilization process. *Microbial cell factories*, *14*, 41. https://doi.org/10.1186/s12934-015-0222-8

13. Gray D. (2001). Overview of protein expression by mammalian cells. *Current protocols in protein science*, *Chapter 5*(1), Unit5.9. https://doi.org/10.1002/0471140864.ps0509s10

14. Vieira Gomes, A. M., Souza Carmo, T., Silva Carvalho, L., Mendonça Bahia, F., & Parachin, N. S. (2018). Comparison of Yeasts as Hosts for Recombinant Protein Production. *Microorganisms*, *6*(2), 38. https://doi.org/10.3390/microorganisms6020038

15. Li, L., Huang, J. L., Liu, Q. C., Wu, P. H., Liu, R. Y., Zeng, Y. X., & Huang, W. L. (2004). Endostatin gene therapy for liver cancer by a recombinant adenovirus delivery. *World journal of gastroenterology*, *10*(13), 1867–1871. https://doi.org/10.3748/wjg.v10.i13.1867

16. Winter L.E., Barenkamp S.J. Construction and immunogenicity of recombinant adenovirus vaccines expressing the HMW1, HMW2, or Hia adhesion protein of nontypeable *Haemophilus influenzae*. Clin Vaccine Immunol. 2010;17:1567–1575.

17. Anderson, G. J., M Cipolla, C., & Kennedy, R. T. (2011). Western blotting using capillary electrophoresis. *Analytical chemistry*, *83*(4), 1350–1355. https://doi.org/10.1021/ac102671n

18. Lee, C. S., Bishop, E. S., Zhang, R., Yu, X., Farina, E. M., Yan, S., et.al. (2017). Adenovirus-Mediated Gene Delivery: Potential Applications for Gene and Cell-Based Therapies in the New Era of Personalized Medicine. *Genes & diseases*, *4*(2), 43–63. https://doi.org/10.1016/j.gendis.2017.04.001

19. Wold, W. S., & Toth, K. (2013). Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. *Current gene therapy*, *13*(6), 421–433. https://doi.org/10.2174/1566523213666131125095046

20. Andrew L.Feldman, Steven K. Libutti, in The Cytokine Handbook (Fourth Edition), 2003

21. Zhen-Yu Ding, Yu-Quan Wei, in Recent Advances in Cancer Research and Therapy, 2012