Gene Delivery into Brain Capillary Endothelial Cells Using Antp-Modified DNA-Loaded Nanoparticles

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Our aim is to test the characteristics and expression activity in brain capillary endothelial cells (BCECs) of DNA-loaded nanoparticles before and after the modification with Antennapedia peptide (Antp). Chitosan (CH), polyethylenimine (PEI) and polyamidoamine (PAMAM) were chosen to prepare the nanoparticles; Lipofectamine 2000 served as control. The incorporation efficiency was determined by agarose gel electrophoresis and PicoGreen assay. Transmission electron microscopy was performed to observe the diameter. The expression efficiency was qualified with fluorescence microscopy and quantified with the luciferase assay system. Heparin and DNase I were employed to investigate the stability. The results indicated that the gene products of DNA/CH nanoparticles and DNA/Lipofectamine 2000 complexes whether modified or unmodified with Antp could not be observed within BCECs. The DNA/PEI nanoparticles and DNA/PAMAM nanoparticles could be internalized into BCECs to a certain extent, and the expression efficiency was enhanced significantly with the modification of Antp. The diameter increased from 100 to 200 nm after modification with Antp. The nanoparticles could protect themselves from the displacement of anionic substances and the digestion of DNase I. These observations demonstrated that Antp, polymer and DNA could form stable nanoparticles, and the modification of Antp can enhance the expression efficiency of DNA-loaded nanoparticles in BCECs.

Key words membrane-penetrating peptide; polyethylenimine; polyamidoamine; DNA-loaded nanoparticle; brain capillary endothelial cells

The membrane-penetrating peptides, a class of peptides capable of translocating oligonucleotides across the cellular and nuclear membranes, have been shown to facilitate the homogeneous uptake of oligonucleotides through a non-endocytic, non-degradative, and energy-independent pathway.[1,2] Antennapedia, a *Drosophila* transcription factor and membrane-penetrating peptide, has been reported to bypass the endosome-lysosome pathway and directly enter the cells *via* a fusion mechanism. Antennapedia peptide (Antp), a 16-amino acid long homeodomain corresponding to the third helix of Antennapedia, possesses the translocating properties comparable to that of the entire Antennapedia.[3] Antp has been successfully used to directly modify drugs and increase their amount in the brain, across blood–brain barrier (BBB).[4]

Despite the widely anticipated potential of cerebral gene therapy, currently available gene vectors are unable to cross BBB through a transluminal administration. Brain capillary endothelial cells (BCECs), the main composition of BBB, are well-known for the lack of endocytic function. However, most cationic polymer vectors are internalized into cells only through endocytosis following electrostatic interaction with phospholipids in the cellular membrane, which results in low transfection efficiency to BCECs. Therefore, efficient gene transfection in BCECs requires a particular membrane-crossing mechanism. In the present study, the general DNA-loaded nanoparticles were modified with Antp to enhance the gene transfection efficiency to BCECs.

At present, there has been an absence of published research on the preparation and transfection of Antp-modified DNA-loaded nanoparticles. The nanoparticles prepared simply with Antp and DNA could not be internalized into cells in spite of Antp's membrane-penetrating function.[2] Cationic polymers have been reported to be widely used in transfecting many types of cells *in vitro*,[5—7] however, they are seldom utilized for transportation of genes into primary BCECs. Therefore, three typical cationic polymers, CH, PEI and PAMAM, were chosen for comparison of their abilities in transportation of report genes into primary BCECs. In our research, DNA/polymer nanoparticle complexes were prepared and their physico-chemical characteristics and transfection efficiency, before and after the modification of Antp, were investigated.

Experimental

Materials BCECs were kindly presented from Prof. JN Lou (the Clinical Medicine Research Institute of the Chinese–Japanese Friendship Hospital). The plasmid pEGFP-N2 (Clontech, U.S.A.) and pGL2-Control Vector (Promega, U.S.A.) were purified by QIAGEN Plasmid Mega Kit (Qiagen GmbH, Germany).

Branched PEI (MW 25 kDa) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). CH (MW about 300 kDa) and PAMAM dendrimers of generation 3 was a generous gift from Prof. SK Fu (Department of Macromolecular Science of Fudan University). Antp (Ac-RQIKIWFQNRRMK-WKK-NH_2) were synthesized by the Biochemistry and Cytobiology Research Institute of Life Science Academy, Academia Sinica. Lipofectamine 2000 and DNase I were purchased from the GIBCO and Takara companies, respectively.

Cell Culture BCECs were isolated from cerebral gray matter of BALB/C mouse and routinely cultured as described previously.[8] Briefly, BCECs were expanded and maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, U.S.A.) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 μg/ml epidermal cell growth factor (ECGF), 2 mmol/l L-glutamine, 20 μg/ml heparin, 40 μU/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin, and cultured at 37 °C under a humidified atmosphere containing 5% CO_2. All cells used in this study were between passage 10 and passage 15.

Nanoparticle Preparation Polymers (CH, PEI or PAMAM) and DNA at different weight ratios were adopted to prepare nanoparticles. Briefly, the polymer and Antp stock solutions were underwent serial dilution with distilled water. A DNA solution (100 μg DNA/ml in 50 mM sodium sulfate solution) was added to the polymer solutions and immediately vortexed for 30 s. The prepared DNA/polymer nanoparticle complexes served as controls. Then the Antp solution was added to the above nanoparticle solutions and

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vortexed for another 30 s, forming DNA/Antp/polymer nanoparticle complexes. The preparation temperature was 55 °C for the PEI group (DNA/PEI NP and DNA/Antp/PEI NP) and the CH group (DNA/CH NP and DNA/Antp/CH NP), and room temperature for PAMAM group (DNA/PAMAM NP and DNA/Antp/PAMAM NP).

In order to determine the optimal preparation conditions, 0.7% agarose gel electrophoresis and PicoGreen assay were performed to evaluate the degree of binding between the polymers and DNA at different weight ratios. Independent agarose gel electrophoresis experiments were performed to assess the binding between Antp and DNA. For the PicoGreen assay, nanoparticles were centrifuged at 14000 rpm for 1.5 h, and then the supernatant was quantified by a PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR, U.S.A.) according to the instructions of the manufacturer.

The nanoparticles produced were used for all studies without further modification. Freshly prepared nanoparticles were used in the following experiments.

***In Vitro* Gene Expression** BCECs were seeded in 24-well plates at a density of 5×10^4 cells/well. Cultured for 24 h, the cells reached 50—60% confluence. The cells were then rinsed twice with Hank's solution. Afterwards, the nanoparticle preparation in FCS-free media was added to the cells and the mixture was incubated at 37 °C. Six hours later, the medium, was replaced with a fresh batch containing FCS. After 42 h, fluorescence images were acquired and photographed under a fluorescence microscope (OLYMPUS, IX 71, Japan). For quantitative analysis, a pGL2-Control Vector was applied. At 2 d post-transfection, the medium was removed from the wells. Luciferase activity was quantified by a Luciferase assay system (Promega, Madison, WI, U.S.A.) according to the manufacturer's instruction. Briefly, the cells were lysed in a sufficient volume of the luciferase cell culture lysis buffer. The lysate was vortexed for 15 s, and then centrifuged at $12000\times g$ for 15 s at room temperature. The activity of luciferase in the supernatant was assayed using a chemiluminescence analyzer (BPCL, Beijing, China). Chemiluminescence was standardised to represent protein level in the cell extract (using the Lowry Method to light units/10 s/mg protein). Lipofectamine 2000 served as control, and the transfection protocol was performed according to the manufacturer's instruction.

Transmission Electron Microscopy (TEM) 0.05 ml of the nanoparticles was deposited on a carbon coated copper grid and left to stand for 2 min, then rinsed with distilled water rapidly. The grid was then stained with 10% phosphotungstic acid for 5 min. The samples were examined under an electron microscopy (Hitachi H-600, Japan) immediately afterwards.

Stability of the Nanoparticles against Heparin and DNase I Heparin was added to the nanoparticle solutions containing a specific amount of DNA—with PEI or PAMAM—to yield a final heparin concentration of 40 units/g plasmid DNA. After 20 min incubation, 0.7% agarose gel electrophoresis was performed to evaluate the amount of DNA displaced from the nanoparticles by heparin.

Naked DNA and the nanoparticles were incubated with DNase I to yield a final DNase I concentration of 50 units/g plasmid DNA at 37 °C for 2 h. Each solution was divided equally. One served as control, and the other was added with 3 μl EDTA (0.5 M) immediately to cease DNase degradation. Three microliters SDS (10%, w/v) was added subsequently in order to displace DNA from the nanoparticles. All samples were then placed in an ice bath. Finally, 0.7% agarose gel electrophoresis was performed to evaluate the integrity of DNA bound to the nanoparticles.

Statistical Analysis Statistical analysis was performed by using ANOVA followed by Bonferroni's test. Data were expressed as mean±standard error of mean (S.E.M.).

Results

Preparation of DNA-Loaded Nanoparticles The results indicated that the cationic peptide or polymers could completely bind with DNA when the weight ratio was 1 : 1 for the DNA/Antp complexes (Fig. 1A), 1 : 0.5 for the DNA/polymer nanoparticle complexes (Figs. 1B—D). The weight ratios were 1 : 10 for DNA/CH NP, 1 : 0.5 for DNA/PEI NP, 1 : 3 for DNA/PAMAM NP, while the polymers could completely incorporate DNA.

The PicoGreen assay results were consistent with the electrophoresis ones. The incorporation efficiency was enhanced with the increase of the DNA/polymer weight ratios and reached almost 100% when the weight ratio was 1 : 0.5 (Table 1).

In result, proper weight ratios were selected for transfection experiments: 1 : 0.5, 1 : 5 and 1 : 10 for DNA/CH NP,

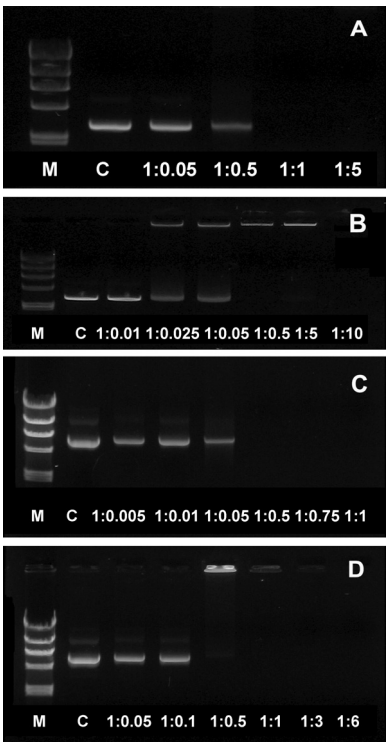

Fig. 1. Agarose Gel Electrophoresis of Complexes at Various Weight Ratios

M: DNA Marker, *Hin*dIII digested; C: naked plasmid DNA. (A) Antp was complexed with plasmid DNA at increasing w/w. Complete exclusion of the dye was achieved above w/w 1 : 1. (B), (C) and (D) represent the agarose gel electrophoresis results of DNA/CH NP, DNA/PEI NP and DNA/PAMAM NP prepared at different weight ratios, respectively.

Table 1. Percentage of Incorporated DNA Encapsulated by Nanoparticles at Various Weight Ratios as Determined with the PicoGreen Assay, Which Quantifies Unbound DNA

DNA/CH	w/w	1 : 0.01	1 : 0.025	1 : 0.05	1 : 0.5	1 : 5	1 : 10
	Encorporation rate (%)	11.26	45.79	50.62	97.67	98.26	99.71
DNA/PEI	w/w	1 : 0.005	1 : 0.01	1 : 0.05	1 : 0.5	1 : 0.75	1 : 1
	Encorporation rate (%)	28.46	53.40	71.22	99.88	99.88	99.88
DNA/PAMAM	w/w	1 : 0.05	1 : 0.1	1 : 0.5	1 : 1	1 : 3	1 : 6
	Encorporation rate (%)	28.50	34.15	97.48	99.62	99.81	99.70

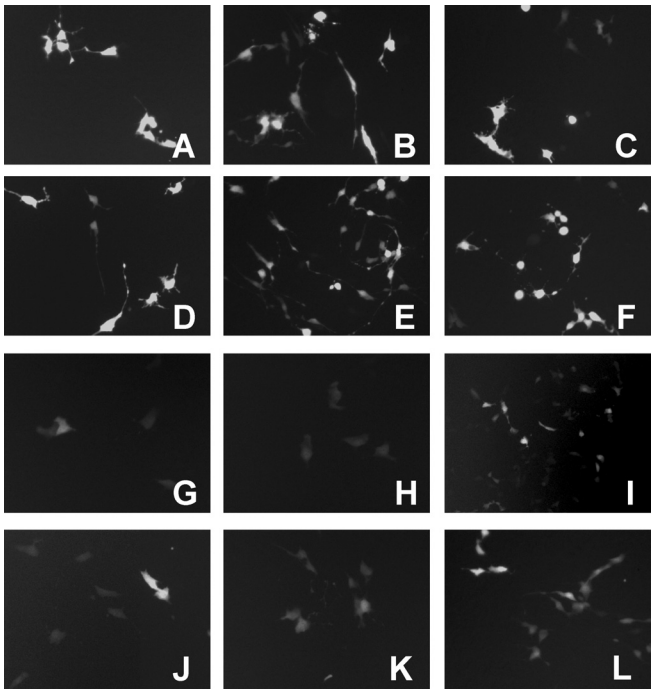

Fig. 2. The Fluorescence Images of GFP Expression in BCECs were Taken 42 h Post-Transfection with the Nanoparticles at Various Weight Ratios

Panels A—C: DNA/PEI NP, 1 : 0.5, 1 : 0.75, 1 : 1, respectively; panels D—F were the corresponding DNA/Antp/PEI NP; panels G—I: DNA/PAMAM NP, 1 : 1, 1 : 3, 1 : 6, respectively; panels J—L were the corresponding DNA/Antp/PAMAM NP. Green: GFP. Original magnification: 200×.

1 : 0.5, 1 : 0.75 and 1 : 1 for DNA/PEI NP, and 1 : 1, 1 : 3 and 1 : 6 for DNA/PAMAM NP, respectively. Furthermore, DNA/Antp ratio was fixed in 1 : 0.5 for all compound nanoparticle preparation.

***In Vitro* Gene Expression** The fluorescence of the green fluorescent protein (GFP) could not be observed in BCECs treated with DNA/CH NP and DNA/Lipofectamine complexes before and after the modification of Antp (results not shown), but could be seen in those transfected with DNA/PEI NP and DNA/PAMAM NP, and enhanced in those exposed to DNA/Antp/PEI NP and DNA/Antp/PAMAM NP (Fig. 2). The optimal DNA/polymer weight ratios were 1 : 0.75 for DNA/PEI NP, 1 : 0.5 : 0.75 for DNA/Antp/PEI NP, 1 : 6 for DNA/PAMAM NP and 1 : 0.5 : 6 for DNA/Antp/PAMAM NP.

The luciferase assay results corresponded with that of fluorescence microscopy. Luciferase could not be detected in BCECs transfected with DNA/CH NP and DNA/Lipofectamine complexes with or without Antp, but could be measured in those transfected with DNA/PEI NP and DNA/PAMAM NP, and the amount of luciferase significantly increased in those exposed to DNA/Antp/PEI NP and DNA/Antp/PAMAM NP compared with the unmodified nanoparticles (Fig. 3). The optimal DNA/polymer weight ratios in terms of luciferase expression were 1 : 0.75 for DNA/PEI NP, 1 : 0.5 : 0.75 for DNA/Antp/PEI NP, 1 : 6 for DNA/PAMAM NP and 1 : 0.5 : 6 for DNA/Antp/PAMAM NP. The luciferase activity in BCECs with 6 h exposure to DNA/Antp/PEI NP (1 : 0.5 : 0.75, w/w/w) was $16.3\times10^6\pm0.982\times10^6$ units/mg protein, being about 2-fold of that of DNA/PEI NP ($7.87\times10^6\pm0.34\times10^6$ units/mg protein), and in those transfected with DNA/Antp/PAMAM NP (1 : 0.5 : 6) was $0.993\times10^6\pm0.073\times10^6$ units/mg protein, being 2.5-fold of that of DNA/PAMAM NP ($0.383\times10^6\pm0.02\times10^6$ units/mg protein). The characteristics of the nanoparticles in the 4 weight ratios were further investigated.

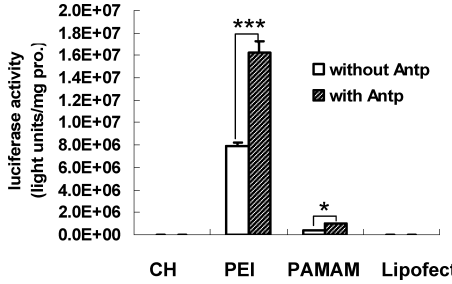

Fig. 3. Transfection Assay for the DNA/Polymer Nanoparticles and DNA/Lipofectamine 2000 Complexes in BCECs

Luciferase activity was measured as described in Experimental 42 h post-transfection and was expressed as light units per mg of protein. $*p<0.05$; $***p<0.001$. Bars represent mean (±S.E.M.); $n=4$.

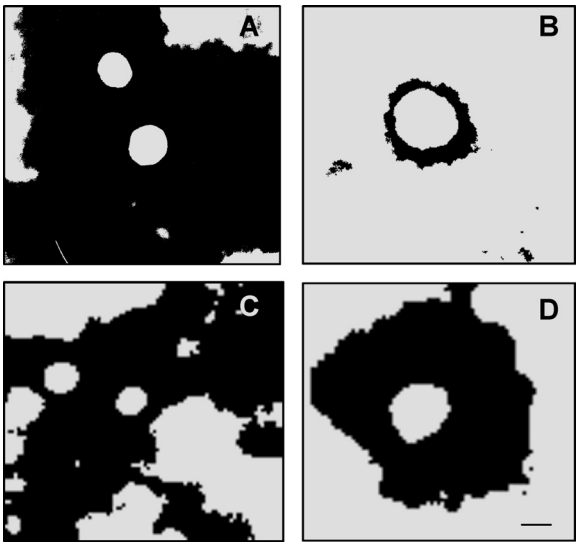

Fig. 4. TEM Images of the DNA/Polymer Nanoparticles with or without Antp

(A) DNA/PEI NP, 1 : 0.75 (w/w), (B) DNA/Antp/PEI NP, 1 : 0.5 : 0.75 (w/w/w), (C) DNA/PAMAM NP, 1 : 6 (w/w), (D) DNA/Antp/PAMAM NP, 1 : 0.5 : 6 (w/w/w). Bar = 100 nm.

Characterization of DNA/Polymer Nanoparticle Complexes The TEM images indicated that the nanoparticles were approximately 100 nm in size for the Antp-unmodified nanoparticles (Figs. 4A, C) and 200 nm for the Antp-modified ones (Figs. 4B, D).

Heparin was adopted to investigate the effect of exogenous anionic substances, which might displace the negatively charged DNA, on the stability of the nanoparticles. As can be seen in Fig. 5A, all nanoparticle types were able to encapsulate DNA and protect it from the displacement by heparin. Naked DNA, as could been seen in Fig. 5B, was digested into small fragments by DNase I while DNA encapsulated by nanoparticles maintained integrity. Thereafter, SDS, which acts as an intense electronegative substance, can displace DNA from the nanoparticles, was added following the DNase treatment. Results showed that the plasmid DNA, recovered from the nanoparticles by SDS treatment, remained

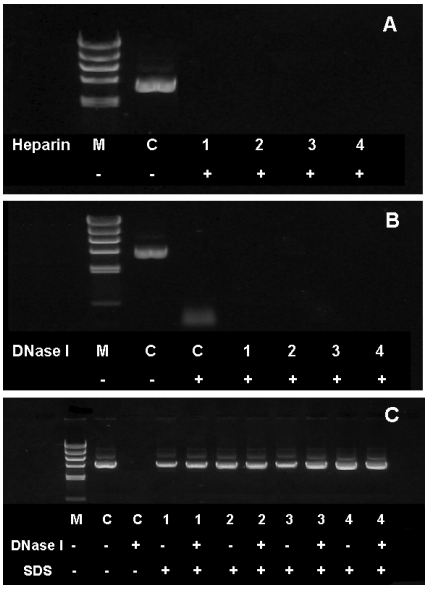

Fig. 5. The Stability Study of the DNA/Polymer Nanoparticles

(A) Effect of heparin on the nanoparticle stability. (B) The nanoparticle stability against DNase I digestion. (C) Determination the integrity of DNA encapsulated by nanoparticles, after DNase treatment. 1: DNA/PEI NP, 1 : 0.75 (w/w); 2: DNA/Antp/PEI NP, 1 : 0.5 : 0.75 (w/w/w); 3: DNA/PAMAM NP, 1 : 6 (w/w); 4: DNA/Antp/PAMAM NP, 1 : 0.5 : 6 (w/w/w); M: DNA Marker, *Hin*dIII digested; C: naked plasmid DNA.

almost completely intact (Fig. 5C).

Discussion

In the present study, Antp, which can penetrate biological membranes in a receptor-independent and energy-independent manner, was employed to modify DNA-loaded nanoparticles transfected to BCECs. Prior to this study, Antp has not been used to modify DNA-loaded nanoparticles for gene transfection. Our research thus focused in screening a range of polymers to form the Antp-modified nanoparticles and investigating their characteristics and expression efficiency in BCECs *in vitro*.

The binding between Antp and DNA was mainly of two mechanisms, electrostatic and homologous interaction.[3] The TEM results indicated that the size of the nanoparticles modified with Antp increased from approximately 100 to 200 nm (Fig. 4). This agrees with the result that the particle size of the liposomes modified with Tat, increased from 150 to 200 nm.[9] This demonstrated the successful modification of Antp in the DNA/polymer nanoparticle complexes.

The expression efficiency of the nanoparticles in BCECs was qualified with fluorescence microscopy and quantified with the luciferase assay system. The results indicated that the expression of DNA in DNA/PEI NP and DNA/PAMAM NP could be observed, and the efficiency was enhanced significantly when the DNA/polymer nanoparticle complexes were modified with Antp (Figs. 2, 3). However, the expression of DNA in DNA/CH NP and DNA/Lipofectamine 2000 complexes with or without Antp could not be observed (data not shown). It suggested that BCECs might not be sensitive to all cationic polymers. And also, antp could only assist the vectors that could transfect cells themselves and enhance their transfection efficiency. There are two mechanisms by which Antp might enhance nanoparticles' expression efficiency. One is Antp's cellular membrane-penetrating function, which can facilitate the DNA/polymer nanoparticle complexes to be uptaken by the cells and enhance the transfection efficiency.[3] The other is it's nuclear membrane-penetrating function, which can assist the DNA/polymer nanoparticle complexes to migrate more easily inside the cells, increase the amount of DNA in the nuclei and further enhance expression efficiency.[10] Above all, Antp was demonstrated to assist the DNA/polymer nanoparticle complexes in entering the cells, to avoid degradation of DNA in the endosomes, and finally to enhance the expression of DNA in BCECs.

In addition, the expression efficiency in BCECs was dependent on DNA/polymer weight ratios. The weight ratios, 1 : 0.75 for DNA/PEI NP, 1 : 0.5 : 0.75 for DNA/Antp/PEI NP, 1 : 6 for DNA/PAMAM NP and 1 : 0.5 : 6 for DNA/Antp/PAMAM NP, were found to generate highest gene expression efficiency in BCECs. The amount of Antp in the nanoparticles also appeared to have an impact on the expression efficiency (data not shown). This agreed with the observation that some fusion peptides such as a pH sensitive fusion peptide (GALA) in the liposomes are able to generate highest transfection efficiency at specific ratios.[11]

Some organic anionic ions such as free fatty acids under physiological conditions may displace the negatively charged DNA from the nanoparticles. Therefore, heparin was adopted to investigate the effect of the anionic substances on the stability of the nanoparticles. As could be seen in Fig. 5A, nanoparticles were demonstrated to be stable enough to be displaced by heparin. Moreover, they could protect DNA from degradation by DNase I (Figs. 5B, C). This indicated that under physiological conditions, a formulation where the nuclease concentration is markedly lower than the tested concentration, would render a significant protection to plasmid DNA.

In summary, a novel complex Antp-modified gene delivery vector was constructed in this research. It was demonstrated to efficiently enhance the expression of DNA in BCECs. The mechanism of the enhancement in transfection and expression efficiency of the nanoparticles with the modification of Antp in BCECs is undergoing further investigation.

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