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Lab Note

Preparation of recombinant human canstatin using transgenic *Dunaliella salina*

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Canstatin, which possesses a significant inhibition effect on the migration of endothelial cells and a strong anticancer effect [1,2], has been applied in the treatment of many cancers including human oral, breast, prostate, pancreatic, and colorectal [3–7]. However, because the expression of bioactive recombinant canstatin is very low using the current expression systems, e.g. prokaryotic *Escherichia coli* expression system [6], its application has still been limited to clinical trials. Several eukaryotic cell expression systems have been exploited for canstatin production, such as *Bombyx mori* cells [8] and *Drosophila melanogaster* S2 cells [9], but they also have a lot of disadvantages, for example, high culture cost, poor yield, and difficulty in purification. Therefore, it is necessary and urgent to develop an optimal expression system for the large-scale production of the recombinant canstatin.

Transgenic *Dunaliella salina* (UTEX-1644) system as a novel potential bioreactor [10,11] is an optimal alternative for the production of the recombinant canstatin due to the following advantages: (i) this expression system has the potential for large-scale production of exogenous proteins; (ii) *D. salina* has been extensively used in industrial and pharmaceutical areas owing to the capability of accumulating valuable fine components such as carotenoids, vitamins, minerals, and proteins; (iii) *D. salina* cells themselves are natural protoplasts, and can be easily transformed and cultured; (iv) eukaryotic *D. salina* cells have the post-transcriptional and post-translational modifications for the production of bioactive proteins. In the present study, therefore, we tried to develop a novel eukaryotic expression system for the recombinant canstatin by using transgenic *D. salina*, and this system will provide a new, safe, and environmental protection platform for the large-scale production of human recombinant canstatin.

The main materials used in this study were as follows: plasmids pBI221-bar and pUΩ-GUS were obtained as a gift from Prof. Yongru Sun (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China). *D. salina* strain UTEX-1644 was purchased from the Algae Culture Collection at the University of Texas (Austin, USA) and cultured in the modified PKS medium under a light intensity of 50 μmol photon m^{-2} s^{-1} with a 12/12-h light/day [12].

To amplify the *canstatin* gene, the total RNA of human placental tissues was isolated. The Primer 1 (5′-ACTCCCGGGGTCAGCATCGGCTACCTCCT-3′) and Primer 2 (5′-CCGAGCTCTCA**ATGGTGATGGTGATGGTG** CAGGTTCTTCATGCACAC-3′) were designed in which the *Sma*I and *Sac*I sites (underlined) were introduced respectively (the bold sequence represents the sequence of His_6). Using the above RNA and primers, the human *canstatin* gene was amplified by reverse transcription–polymerase chain reaction (RT–PCR) and the results showed that a specific fragment of 700 bp was successfully amplified. The results of sequencing indicated that the amplified DNA fragment was completely identical to the nucleotide sequence reported in GenBank. Then, the *canstatin* gene was inserted into pMD18-T (TaKaRa, Dalian, China) to yield a new plasmid pMD18-T-Can for plasmid propagation and DNA sequencing. Subsequently, the *canstatin* fragment cut from the pMD18-T-Can by digestion of endonucleases was inserted into pUΩ-GUS to generate a novel vector pUΩ-Can. After being confirmed by digestion of double enzymes, the pUΩ-Can was recovered and further connected with the bar box to generate a eukaryotic expression vector pUΩ-Can-Bar.

pUΩ-Can-Bar vectors were transformed into the *D. salina* cells using the glass beads method [12], and then the transformed *D. salina* cells were incubated for 24 h under dim light conditions. After the transformed *D. salina* cells were incubated for 2 weeks on a 1% agar plate containing 3 mg/l of phosphinothricin (PPT), the individual positive colonies were observed, among which four colonies were picked out and inoculated into the liquid medium with 3 mg/l of PPT for further selection. The resistant test of the transformants demonstrated that all the transformants survived from the following

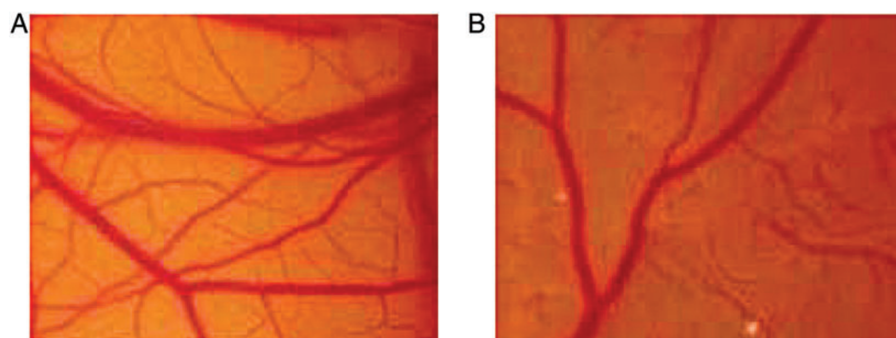

Figure 1. Inhibition of the newly formed blood vessels by recombinant human canstatin proteins on CAM (A) No anti-angiogenesis was observed in the negative control embryos treated with the filter paper soaked in phosphate buffered saline (PBS). (B) A large number of newly formed blood vessels were significantly suppressed in the embryos treated with the filter paper soaked in the solution containing recombinant human canstatin proteins.

repeated PPT selections and could be stably maintained for at least 3 months. Moreover, the results of RT−PCR analysis showed that the *canstatin* gene with a fragment of 700 bp was detected in the four transformants but not in the negative control group. PCR-southern blot analysis also demonstrated that a hybridization signal was detected in the four transformants, which corresponded to the hybridization signal of the PCR fragment, but not in the negative control group. Cells treated with the same transformation protocol without the plasmid pUΩ-Can-Bar were used as negative controls.

To analyze the recombinant canstatin protein activities, the total proteins of the negative group and transformants were extracted, respectively. After staining with Coomassie blue, the recombinant canstatin proteins were detected in the transformants on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (**Supplementary Fig. 1A**, Lanes 2 and 4), but not in the negative control group (**Supplementary Fig. 1A**, Lanes 1 and 3). The inserted canstatin fragments yielded a mature protein of 236 amino acids comprising the 227 amino acids of canstatin, 6 amino acids of histidine, and 3 extra amino acids. The recombinant canstatin has a molecular mass of ∼26 kDa, which may be due to the recombinant protein expressed in *D. salina* as a fusion protein with a histidine tag [8,15]. After being purified with a Ni-NTA resin purification kit (Qiagen, Hilden, Germany), a total of 84 μg purified canstatin with a molecular weight of ∼26 kDa was obtained from the 100 ml cultures of *D. salina* that was further identified by western blot analysis (**Supplementary Fig. 1B**). In contrast to the published reports [6,8], however, the amount of the purified canstatin was lower in the present study. Therefore, an endogenous promoter of *D. salina* cells [15] was cloned and inserted in order to improve the expression level of canstatin proteins. Future studies should be done including the construction of high-efficient expression vectors for *D. salina* and screening of the transformants with stable, unvarying, and high expression of canstatin.

Although the amount of the recombinant canstatin expressed by the transgenic *D. salina* was lower than that by *B. mori* cells [8] or *D. melanogaster* S2 cells [9], the transgenic *D. salina* system will become a better alternative for the production of the recombinant canstatin in the future after a series of studies focusing on expression levels are carried out.

To investigate the anti-angiogenic activity of recombinant canstatin, the chicken embryo chorioallantoic membrane (CAM) assay was carried out as described previously [13,14]. The filter papers of 1.5 mm^2 immersed in a series of gradient recombinant canstatin proteins at concentrations of 6, 12, 18, 24, and 30 μg, respectively, were taken out and air-dried. After the air chambers of the 5-day-old chicken embryos were carefully removed, the filter papers were implanted onto the CAM and then incubated for 72 h at 37°C. The results of the CAM assay showed that the large number of newly formed blood vessels were significantly inhibited by canstatin and no obvious inflammation happened (**Fig. 1B**), but anti-angiogenesis was not observed in the control embryos (**Fig. 1A**).

We further demonstrated that inhibition of angiogenesis of the chicken embryo by a range of 6−30 μg canstatin proteins was in a dose-dependent manner as shown in the chicken embryo CAM assay. Moreover, a near-complete inhibition of newly formed blood vessels on CAM was observed when canstatin protein of 24 μg/CAM was used (**Fig. 2**), and the ED_{50} for the purified recombinant human canstatin was ∼7.11 μg/CAM. Anti-angiogenesis effect of the human canstatin proteins prepared by transgenic *D. salina* was higher than those derived from *Escherichia coli* [14], suggesting that the recombinant human canstatin proteins may be used in the anti-angiogenic therapy for human cancers.

In summary, the recombinant human canstatin with anti-angiogenesis activity has been successfully prepared by transgenic *D. salina*, which provides a novel approach for the large-scale production of the angiogenesis inhibitor for cancer therapy. In order to obtain the high-efficienct expression of foreign gene in *D. salina*, the following aspects should be strengthened in future studies, including the

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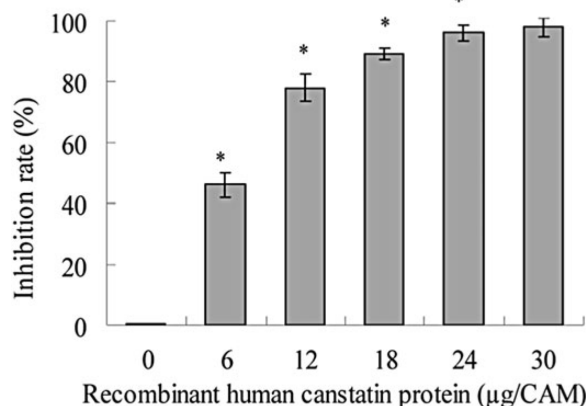

Figure 2. Inhibition of the recombinant human canstatin at different concentrations of proteins The data are shown as the percentage of CAM number relative to avascular zones. $*P < 0.05$, compared with the negative control embryos treated with the filter paper soaked in PBS.

genome sequencing of *D. salina*, exploitation of the novel green selective marker, and establishment of effective molecular toolkits and the standard transformation procedures [16]. Moreover, to solve the problem of low expression in a *D. salina* bioreactor, some strategies could be used to increase the protein yields, such as codon optimization [17], transformation-associated genotypic modifications, reduction of sensitivity to proteases, fusion of recombinant products to native proteins [18], and development of *D. salina* chloroplast expression system.

Supplementary data

Supplementary data is available at *ABBS* online.

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