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Expression and *In Vitro* Activity of Recombinant Canstatin in Stably Transformed *Bombyx mori* Cells

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We describe the expression of recombinant canstatin from stably transformed *Bombyx mori* Bm5 (Bm5) cells. Recombinant canstatin was secreted into a culture medium with a molecular mass of approximately 29 kDa. Densitometric scanning showed that the secreted canstatin accounted for approximately 91% of the total canstatin production. Recombinant canstatin was also purified to homogeneity using a simple one-step Ni-NTA affinity fractionation. The identity of the purified protein was confirmed as human canstatin by nano-LC-MS/MS analysis. Purified recombinant canstatin inhibited human endothelial cell proliferation in a dose-dependent manner. The concentration at half-maximum inhibition (ED_{50}) for recombinant canstatin expressed in stably transformed Bm5 cells was approximately 0.64 µg/ml. A maximum production level of 11 mg/l recombinant canstatin was obtained in a T-flask culture of Bm5 cells after 6 days of incubation.

Keywords: Recombinant canstatin, *Bombyx mori*, expression, purification, *in -vitro* activity

Angiogenesis is the growth of new blood vessels from preexisting vasculature and is necessary for tumor growth and metastasis. Several angiogenic growth factors are secreted into the tumor microenvironment that can induce endothelial cell proliferation, migration, and capillary tube formation. Intervention of any one of these processes can affect angiogenesis and, consequently, inhibit tumor growth [2]. Certain factors, including endostatin, tumstatin, and canstatin are regarded as endogeneous inhibitors of angiogenesis *in vivo* [5, 8, 10].

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Canstatin is the 24 kDa NC1 domain of the α2 chain of type IV collagen. Canstatin inhibits endothelial cell migration and tube formation [3–5]. Canstatin also inhibits Akt activation and induces Fas-dependent apoptosis in endothelial cells [11]. In addition, canstatin inhibits tumor growth in mouse models [5] and is a potent inhibitor of angiogenesis with a distinct antitumor activity [5, 9].

An efficient expression system needs to be developed to produce the large quantities of soluble canstatin necessary for early clinical trials. Recently, canstatin expression was reported in *Escherichia coli*, 293 embryonic kidney cells [5], and dipteran *Drosophila melanogaster* S2 cells [7]. In this report, we describe stable expression of the cDNA for human canstatin in lepidopteran *Bombyx mori* Bm5 cells and purification of recombinant canstaitn using metal chelate affinity fractionation. We also describe the *in vitro* activity of recombinant canstatin derived from stably transformed Bm5 cells.

Materials and Methods

Cell Line, Plasmids, and Enzymes

Bombyx mori Bm5 cells were grown at 27°C in a T-25 culture flask (Nunc, Roskilde, Denmark) in TC-100 insect medium (Sigma, St. Louis, MO, U.S.A.) containing 10% (v/v) FBS (fetal bovine serum; Hyclone, Waltham, MA, U.S.A.). The 3.3-kb pIZT/V5-His plasmid (Invitrogen, Carlsbad, CA, U.S.A.) contained a constitutive OpIE2 promoter, a V5 epitope tag, a polyhistidine region, and a zeocin resistance gene under control of the EM7 promoter. The plasmid containing cDNA, which encodes human canstatin, was pMT/BiP/Can-V5-His [7]. *E. coli* DH5α was used as the primary host for constructing and propagating plasmids. *E coli* cells were routinely grown with agitation at 37°C in LB medium [1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.3)] containing 50 µg/ml of ampicillin. We used DNA restriction enzymes from Promega (Madison, WI, U.S.A.) and Takara (Shiga, Japan) according to the manufacturer's instructions.

Construction of Expression Plasmids

The human canstatin sequence attached to the *Drosophila* BiP signal sequence was amplified from plasmid DNA, pMT/BiP/Can-V5-His, by PCR using oligonucleotide primers. The sense primer was 5'-GGTACCATGAAGTTATGCATATT-3' and the antisense primer was 5'-GCGGCCGCCCAGGTTCTTCATGC-3'. The amplified canstatin sequence was then inserted into the pGEM-T vector (Promega) to yield pGEM-T-BiP-Can, and the construct was verified by DNA sequencing. PCR was performed on a Thermal Cycler (Applied Biosystems, Foster City, CA, U.S.A.) using a PCR mix (Takara) in a volume of 50 µl. pIZT/BiP/Can-V5-His was constructed by inserting the KpnI-NotI fragment of pGEM-T-BiP-Can between the KpnI and NotI sites of pIZT/V5-His (Fig. 1). The proper orientation and reading frames of the insertions in pIZT/BiP/Can-V5-His were confirmed by both restriction enzyme mapping and DNA sequencing.

Stable Transformation

Exponentially growing Bm5 cells were transfected with the pIZT/BiP/Can-V5-His using the lipofectamine method. For preparation of the transfection medium, plasmid DNA and the lipofectamine reagent (Invitrogen) were diluted separately in FBS-free TC-100 medium, and then mixed at a 1:5 ratio. The transfection medium was incubated at room temperature for 45 min, and then transferred into 6-well plates pre-seeded 2 h earlier with Bm5 cells in FBS-free TC-100 medium. After 24 h incubation, the cells were incubated for 5 additional days without drug selection in TC-100 medium containing 10% FBS and 200 µg/ml zeocin (Invitrogen). The selective medium was replaced every 5 days, and stably transformed polyclonal cell populations were isolated after 4 weeks of selection with zeocin. Zeocin was maintained continuously in the media after the selection procedure.

Cell Culture and Analysis of Gene Expression

Stably transformed Bm5 cells expressing recombinant canstatin were grown at 27°C in Nunc (T-25) flasks in 5 ml of TC-100 medium containing 10% FBS and 200 µg/ml zeocin. Stably transformed Bm5 cells were cultured in multiple flasks to analyze cell growth and canstatin expression.

The cultures were centrifuged at 3,000 rpm for 5 min and the supernatants were used to identify extracellular recombinant proteins.

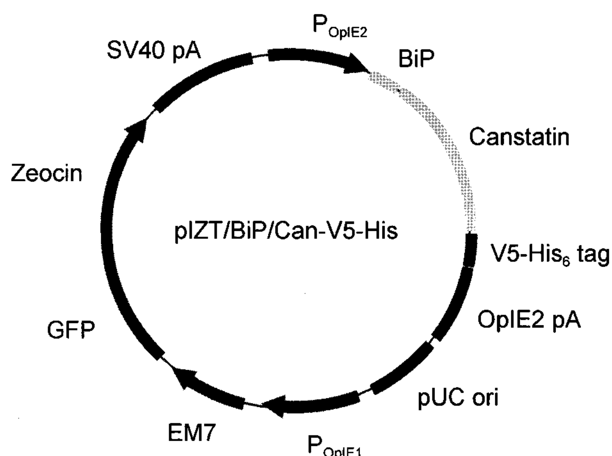

Fig. 1. Schematic representation of the expression plasmid pIZT/BiP/Can-V5-His.

The cell fraction was incubated with rocking for 30 min in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 µg/ml of PMSF, 1 µg/ml of aprotinin, and 1% Triton X-100], and subjected to three freeze-thaw cycles of freezing at −70°C followed by incubation at 37°C in a water bath. The cell extracts were centrifuged at 14,000 rpm for 15 min to remove cell debris, and the supernatant was used to identify intracellular recombinant proteins. A mixture of the intracellular and extracellular fractions was used to analyze total protein production, unless specified otherwise.

SDS-PAGE and Western Blot Analysis

Protein samples that were separated by electrophoresis on 12% polyacrylamide-SDS gel [6] were visualized by silver staining [12] and subjected to Western blot analysis. The electrophoresed proteins on the gel were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ, U.S.A.), blocked with 3% skim milk, incubated with mouse anti-V5 (1:1,000 dilution in TBS; Invitrogen), and probed with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (1:1,000 dilution in TBS; Sigma). The membranes were washed and BCIP/NBT solution (Amresco, Solon, OH, U.S.A.) was added. The reaction was quenched with distilled water.

Purification of Recombinant Canstatin

All steps were performed at 4°C. Polyhistidine-tagged canstatin was purified using Ni-NTA resin (Qiagen, Valencia, CA, U.S.A.) in a chromatography column (Novagen, Madison, WI, U.S.A.), according to the manufacturer's instructions. The resin was equilibrated with a binding buffer containing 10 mM imidazole, and then poured into the column. The medium fraction of the culture was then slowly applied to the column containing the equilibrated resin. Weakly bound proteins were washed from the resin using the binding buffer, and contaminating proteins bound to the resin were removed by increasing the imidazole concentration to 30 mM. The recombinant polyhistidine-tagged protein product was finally eluted in the buffer containing 200 mM imidazole. Fractions containing canstatin were dialyzed in 1×HBSS (pH 7.4) to remove the imidazole. Protein concentrations were determined using a Bradford Protein Assay Kit (Bio-Rad, Hercules, CA, U.S.A.) with bovine serum albumin as a standard.

Nano-Liquid Chromatography–Tandem Mass Spectrometry (nano-LC-MS/MS) Analysis

Purified canstatin was electrophoresed on SDS-PAGE, stained with Coomassie blue, and the appropriate band was excised from gel with a sterile scalpel. Peptides were analyzed by nano-LC-MS/MS at Yonsei Proteome Research Center (http://www.proteomix.org). Nano-LC-MS/MS analysis was performed on an Agilent 1100 Series nano-LC and an LTQ-mass spectrometer. The capillary column used for LC-MS/MS analysis (150 mm×0.075 mm) was obtained from Proxeon (Odense C, Denmark) and slurry packed in-house with 5 µm, 100 Å pore size Magic C18 stationary phase (Michrom Bioresources, Auburn, CA, U.S.A.). The mobile phase A for the LC separation was 0.1% formic acid in deionized water and the mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was set up to give a linear increase from 5% B to 35% B in 50 min and from 40% B to 60% B in 20 min and from 60% B to 80% B in 5 min. The flow rate was maintained at 300 nl/min after splitting. Mass spectra were acquired using data-dependent acquisition with

full mass scan (400–1,800 m/z) followed by MS/MS scans. Each MS/MS scan acquired was an average of one microscan on the LTQ. The temperature of the ion transfer tube was controlled at 200°C and the spray was 1.5–2.0 kV. The normalized collision energy was set at 35% for MS/MS. Sequest software was used to identify the peptide sequence. For high confidence limits, deltaCn≥0.1, Rsp≤4, and Xcorr≥1.5 with charge state 1+, Xcorr≥2.0 with charge state 2+, and Xcorr≥2.5 with charge state 3+, and a peptide probability >0.1 were used as cutoff values for protein identification. Peptides were variably oxidized at methionine residues and variably carboxyamidomethylated and carboxymethylated at cysteine.

Human Umbilical Vein Endothelial Cell Proliferation Assay
The endothelial cell proliferation assay procedure was a modification of the method of O'Reilly *et al* [10]. Human umbilical vein endothelial (HUVEC) cells (Clonetics, San Diego CA, U.S.A.) were maintained in EGM-2 containing 2% FBS, 12 μg/ml of bovine brain extract, 10 ng/ml of human epidermal growth factor, 1 μg/ml of hydrocortisone, 50 μg/ml of gentamycin, and 50 μg/ml of amphotericin, as suggested by the manufacturer (Clonetics). Approximately 2×10^4 cells in 0.5 ml of EGM-2 were added to each well of gelatinized 24-well plates, followed by incubation at 37°C (in 5% CO_2) for 24 h. The medium was replaced with 0.25 ml of fresh EGM-2 (described above) containing antibiotics, and different concentrations of recombinant canstatin were added to each well. After 30 min of incubation, the cells were stimulated with 1 ng/ml basic fibroblast growth factor (bFGF), and EGM-2 containing antibiotics was added to achieve a final volume of 0.5 ml. After 48 h of incubation, the cells were trypsinized and counted using a hemacytometer. To ensure that observed inhibition was not due to detachment of HUVEC cells from the plate, all wells were examined under an inverted microscope for evidence of cell detachment prior to cell counting. The endothelial cell proliferation assay was performed three times. The concentration at half-maximum inhibition (ED_{50}) was estimated by a probit analysis [1].

RESULTS AND DISCUSSION

Expression of Recombinant Canstatin

We examined the expression of recombinant canstatin in stably transformed *Bombyx mori* Bm5 (Bm5) cells. Gene expression at 5 days after incubation was analyzed by Western blot analysis. As shown in Fig. 2, the 29 kDa recombinant canstatin protein was detected in transformed Bm5 cells carrying pIZT/BiP/Can-V5-His, by Western blot analysis with mouse anti-V5 antibodies (Invitrogen). The molecular size approximated to the predicted molecular mass (29.5 kDa) of recombinant canstatin. Based on the vector construct, cleavage of the BiP signal peptide yielded a mature protein of 268 amino acids that contained the 227 amino acids of canstatin, the 23 amino acids of the C-terminal tag of V5 and His_6, and 18 amino acids of extra sequences derived from the cloning site. Recombinant canstatin was present in the intracellular and extracellular (medium) fractions of transfected Bm5 cells. Densitometric scanning showed that the secreted canstatin (i.e., canstatin in the medium) accounted for approximately 91% of the total canstatin production. The stably transformed Bm5 cells efficiently secreted the expressed canstatin into the extracellular (medium) fraction. Recombinant canstatin was not detected in either the cellular or medium fractions of nontransfected Bm5 cells.

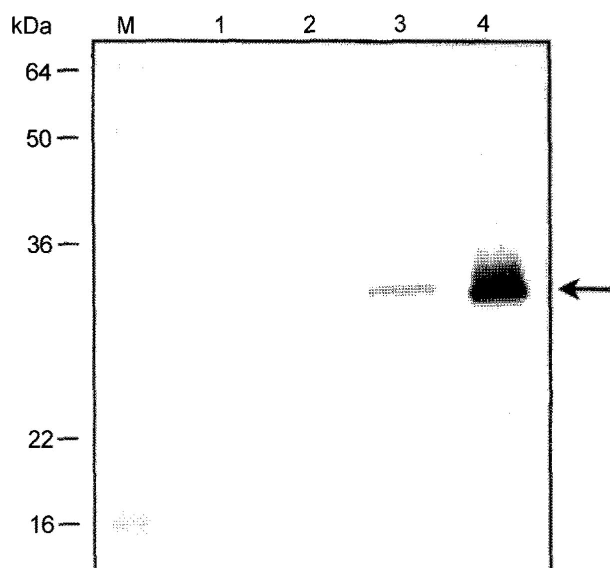

Fig. 2. Western blot analysis of nontransfected and stably transformed Bm5 cells.
The numbers on the left and the arrow indicate the molecular mass markers (kDa) and the recombinant canstatin protein, respectively. Lane 1, cellular fraction of nontransfected cells; lane 2, medium fraction of nontransfected cells; lane 3, cellular fraction of stably transformed Bm5 cells; and lane 4, medium fraction of stably transformed Bm5 cells.

Purification and Identification of Recombination Canstatin

Recombinant canstatin protein in the extracellular fraction of stably transformed Bm5 cells was purified by Ni-NTA affinity chromatography. The purity of the protein was analyzed using SDS-PAGE and silver staining (Fig. 3A). Western blot analysis further confirmed the identity of the purified protein (Fig. 3B). A total of 300 μg of purified polyhistidine-tagged canstatin (canstatin-V5-His_6) was obtained from the medium fraction of a 100-ml culture. The identity of the purified protein was also confirmed by nano-LC-MS/MS. As shown in Fig. 3C, five of the tryptic peptides resolved by LC-MS/MS matched the amino acid sequence of human canstatin with a coverage of 33%.

Human Umbilical Vein Endothelial Cell Proliferation Assay

Canstatin, which is an angiogenesis inhibitor, suppresses endothelial cell growth *in vitro* [9, 11]. The effect of purified recombinant canstatin on suppression of bFGF-stimulated endothelial cell proliferation was examined to determine whether this protein is biologically active. Recombinant canstatin inhibited proliferation of human

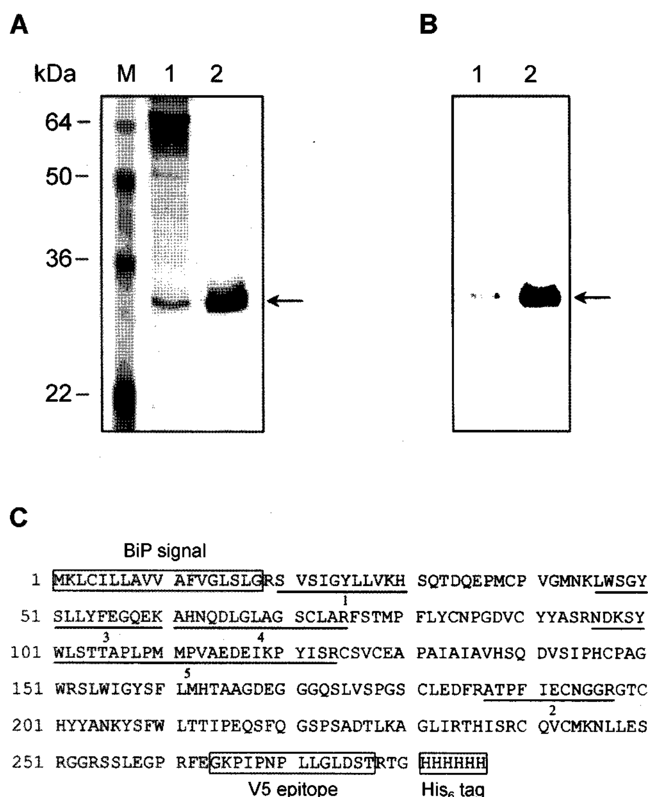

Fig. 3. SDS-PAGE (**A**) and Western blot analysis (**B**) of the purification of His-tagged canstatin from the medium fraction of a stably transformed Bm5 cell culture.

M indicates the molecular mass marker. Before and after affinity purification of the medium fraction of stably transformed Bm5 cells are shown in lanes (1) and (2), respectively. The arrows indicate the recombinant canstatin protein. **C.** Identification of the purified recombinant canstatin. The 29 kDa band was excised from a Coomassie blue stained gel and digested with trypsin. Tryptic peptides resolved by LC-MS/MS are shown in underline and numbered as 1 to 5. The signal sequence, VP5 epitope, and expression tag are highlighted in a pane.

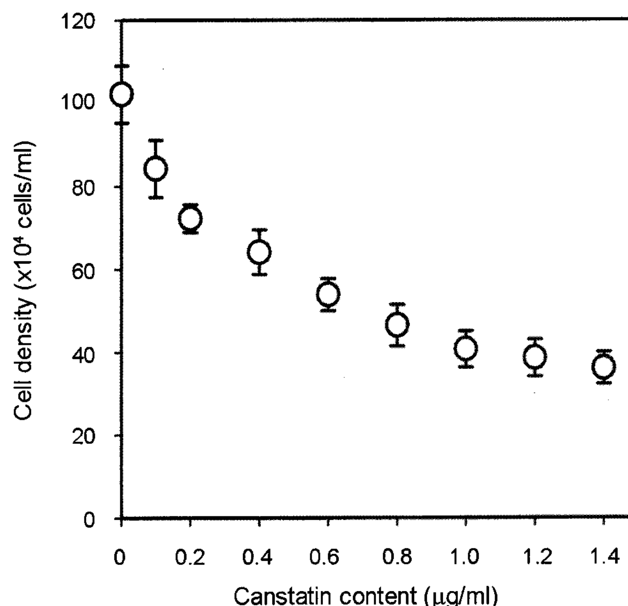

Fig. 4. Inhibition of endothelial cell proliferation by recombinant canstatin.

Purified recombinant canstatin was added with 1 ng/ml bFGF to human umbilical vein endothelial cells in a 48 h proliferation assay.

umbilical vein endothelial cells in a dose-dependent manner (Fig. 4). The concentration at half-maximum inhibition (ED_{50}) for recombinant canstatin was approximately 0.64 μg/ml. This ED_{50} value is higher than the concentrations (0.5 and 0.37 μg/ml) reported for recombinant canstatin proteins derived from *E. coli* [5] and *Drosophila melanogaster* S2 cells [7], respectively. Our result shows that a biologically active form of recombinant canstatin expressed from Bm5 cells exhibits an antiproliferative action on human endothelial cells.

Time-Course Expression

Fig. 5 shows the time-course changes in cell density and recombinant cantatin expression during T-flask cultures of stably transformed Bm5 cells. The initial cell density was 0.9×10^6 cells/ml. The maximum cell density was 2.7×10^6 cells/ml after 6 days of cultivation. The canstatin accumulation peaked 6 days after incubation, when recombinant canstatin production was estimated to be about 11 mg/l by comparison with a purified canstatin control on a Western blot. On a specific-productivity basis, canstatin production was 6.8 μg/10^7 cells/day. Our experiment suggest that alternative lepidopteran insect cell lines such as *Bombyx mori* Bm5 cells may be used as effectively as dipteran insect cells, *D. melanogaster* S2 cells [8.5 μg/10^7 cells/day], for recombinant canstatin production [7]. However, the expression level in our system could not be compared directly with the level from 293 embryonic kidney cells [5], owing to a lack of appropriate data. Bm5 cells offer advantages over 293 embryonic kidney cells as follows: Bm5 cells grow readily in suspension to high cell densities at room temperature and Bm5 cells are maintained easily because they require neither trypsin treatment for splitting nor a CO_2 supply. Our Bm5 cell-expression system expresses recombinant canstatin efficiently and this system may be used as an alternative to the baculovirus-insect cell system. Interestingly enough, stably transformed Bm5 cells were recovered from a single transfection after 4 weeks of zeocin selection. Therefore, the time required for constructing transformed Bm5 cells is reasonably comparable to the baculovirus-insect cell system, in which recombinant baculoviruses are obtained after about 3–4 weeks by using a commercial kit (Invitrogen).

In summary, we report the expression of canstatin in a stably transformed *Bombyx mori* Bm5 cell system. Our findings demonstrate that the purified, biologically active form of recombinant canstatin expressed from Bm5 cells has antiproliferative action on bFGF-stimulated human endothelial cells. The high level of functional expression

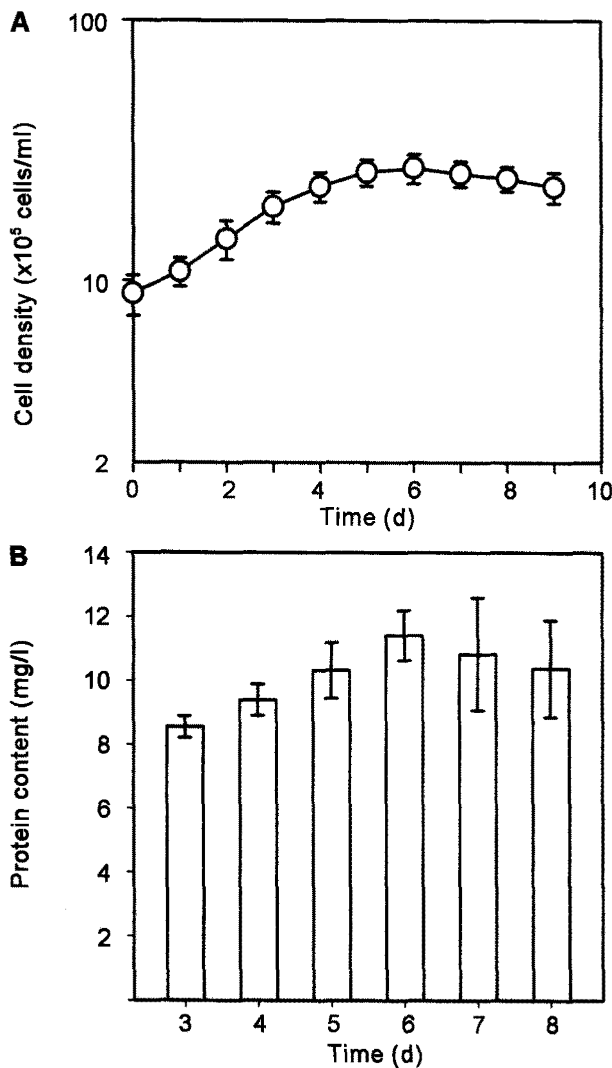

Fig. 5. Time-course changes in cell growth and recombinant canstatin expression during culture of transformed Bm5 cells.
A. The cell concentrations are plotted against the incubation time. **B.** Recombinant canstatin expression (as confirmed by Western blot analysis) at incubation times of 3, 4, 5, 6, 7, and 8 days is shown in lanes 1, 2, 3, 4, 5, and 6 respectively.

could provide a convenient source of recombinant canstatin for research into anticancer therapeutics.

Acknowledgments

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