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High level expression and purification of recombinant PEX protein in cultured skeletal muscle cell expression system ☆

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Abstract

Large quantities of recombinant proteins are needed for specific therapeutic and diagnostic applications. To achieve high-level expression in eukaryotic cells, the choice of cell line as well as the expression vector is critical. In this report, we demonstrate that a combination of the skeletal muscle cell line, QM7 and a cytomegalovirus promoter-based expression vector can achieve high-level expression of secretory recombinant proteins in eukaryotic cells. We also screened a serum-free medium containing 3 μg/ml insulin suitable for QM7 differentiation and identified a very potent signal peptide from MMP9, which effectively directs secretion of heterologous proteins. The C-terminal hemopexin-like domain of MMP-2, PEX, a powerful candidate for the treatment of diseases associated with neovascularization was expressed in QM7 cells with bioactivity. This skeletal muscle cell-based system may be employed for the production of human proteins of special interests, such as those for structural determination or therapeutical development.

Keywords: QM7; Expression system; Skeletal muscle; PEX; Signal peptide; MMP9

Large quantities of recombinant proteins are needed for specific therapeutic and diagnostic applications. For this purpose, various expression systems have been developed with the aim of producing, at reasonable cost and effort, bioactive proteins. *Escherichia coli*-based expression systems have been the primary choice because of simplicity and high yield [1–3]. However, recombinant proteins expressed in the cytoplasm of bacteria are often misfolded as insoluble inclusion bodies and are therefore inactive. Several eukaryotic systems are being developed and evaluated as alternatives to produce soluble and bioactive proteins. The insect cells/baculovirus-based expression system has been used successfully to express many mammalian proteins with functional activities [4,5]. For many mammalian proteins, especially those secreted and modified posttranslationally, a more compatible expression system is highly desirable because proper folding or modification can only be provided with closely related cells. However, the level of expression in mammalian cell-based systems tends to be relatively low and unsatisfactory for practical purpose. Since the host cell line is the ultimate factor restricting the development of expression systems, it is important to identify a novel host cell line improving on the cell lines existing now.

QM7 cells belonged to myogenic cell lines derived and isolated from the quail fibrosarcoma cell line QT6 [6]. In most respects, QM cells closely resemble their mammalian counterparts. Differentiation of QM cells is serum dependent; following removal of serum, cells withdraw from the cell cycle, fuse into myotubes. Myotubes can maintain survival for a long time in low-serum medium and still synthesize proteins. This characteristic endows the cell with several advantages for recombinant protein expression. In

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this paper, we describe a novel strategy to produce a large quantity of recombinant protein in a skeletal muscle cell-based system. Briefly, we have developed a serum-free medium containing 3 μg/ml insulin suitable for QM7 cells differentiation and recombinant protein purification. We have identified a very potent signal pepetide from MMP9, which effectively directs secretion of heteroprotein in QM7 cells. We used QM7 cells to express the C-terminal hemopexin-like domain of MMP-2, PEX, an endogenous angiogenesis inhibitor and a very promising candidate for the treatment of diseases associated with neovascularization [7]. This expression system offers simplicity and economy for large-scale productions of mammalian proteins in native environment.

Materials and methods

Cell culture. QM7 cells were gifts from Dr. Parker B Antin (University of Arizona). The cells were seeded at the density of 10,000/cm^2 in Medium 199 (GIBCO-BRL), supplemented with L-glutamine (1 mM), tryptose phosphate broth (0.2%), fetal bovine serum (10%), and streptomycin/penicillin (50 U/ml). Twenty four hours to 48 h after plating, when cells were completely confluent, culture cells were washed with PBS and either shifted to 0.5% fetal bovine serum-containing 199 medium (differentiation medium, DM), or to serum-free medium consisting of 199 medium supplemented with 3 μg/ml insulin (Sigma). The medium was refreshed every 24 h for 7 days. The degree of myogenesis was morphologically assessed at 1d, 2d, 4d and 6d by Giemsa staining.

Construction of plasmids. The eukaryotic expression vector we used was pcDNA3 (Invitrogen) which contains the cytomegalovirus early promoter/enhancer sequence and ampicillin resistance gene. Table 1 shows the sequences of oligonucleotide primers used for construction of different plasmids. The construction of prGHPEX was described previously [8]. In brief, it encodes PEX with a 6× histidine/myc tag attached to the C terminus and a rat growth hormone (rGH) signal sequence fused to the N terminus of PEX in-frame. The sequences coding for signal peptide of mouse IgG κ chain and MMP9 were synthesized with primer A, B and C, D (Table 1), which was cloned into prGHPEX digested with *Hin*dIII and *Bam*HI, resulting in pIgPEX and pM9PEX expression vectors.

Transfection of QM7 cells. Transfections were performed with Lipofectamine Reagent (Invitrogen) according to manufacturer's specifications. For each transfection, 2 μg plasmid DNA and 10 μl lipofectamine were combined in 1 ml serum-free medium and added to 3×10^5 cells in 35 mm culture wells.

Semiquantitative analysis of protein expression levels in QM7 cells. To determine the mRNA expression levels of target genes, semiquantitative RT-PCR was performed. QM7 cells were plated on a 12-mm coverslip at a density of 1×10^5 cells and transfected with various constructs of PEX for 2 h. After 6 h incubation, total cellular RNA was extracted from the cells using TRIzol reagent (Gibco-BRL). One microgram of total RNA was reverse transcribed, and amplified by PCR using primers 5′-TCTGCAAG CACGAC-3′ (sense) and 5′-GCAACCCAACCAGTC-3′ (anti-sense) for PEX. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous reference. The same amount of cDNA was amplified by PCR for GAPDH using the primers 5′-GTGACC CCAGCAACATCA-3′ (sense) and 5′-CCAGTAAGCTTCCCATTC-3′ (anti-sense). The PCR products were electrophoresed on a 2% agarose gel, and visualized with ethidium bromide. The density of each band was measured using a gel-doc image analyzer. The band intensities of PEX were normalized to those of GAPDH in the corresponding sample.

Western blot analysis. The supernatants were collected and cells were harvested and resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40) supplemented with protease inhibitor. The cytoplasmic lysates were sonicated and centrifuged to remove nuclei. Supernatants from growth medium (5 μl) and 10 μg of cytoplasmic lysates were run on an SDS–PAGE and the proteins were transferred to a PVDF membrane. The blot was incubated with monoclonal anti-c-myc antibody 9E10 (Cell Center, University of Pennsylvania, Philadelphia, PA). Secondary antibodies were horseradish peroxidase-conjugated rabbit anti-mouse antibody (Jackson Immunochemicals).The proteins were detected with enhanced chemiluminescence (Amersham Biosciences).

ELISA. For PEX ELISA, 96-well plates were coated with monoclonal anti-c-myc antibody 9E10 at 5 μg/ml in 100 mM sodium bicarbonate buffer, pH 9.0, for 16 h at 4 °C. After washing five times in 0.0002% (w/v) Tween 20 in PBS, the plates were blocked with 5% (w/v) defatted milk in PBS at 37 °C for 2 h. Purified standard protein was diluted to 250 ng/ml, followed by serial two-fold dilutions in PBS. Each well was incubated with 100 μl of sample or standard and agitated at 37 °C for 2 h. After washing five times with 0.0002% (w/v) Tween 20 in PBS, each well was incubated with 100 μl of rabbit anti-pex antibody diluted 1/1500 in 5% (w/v) defatted milk in PBS and agitated at 37 °C for 2 h. After washing five times with 0.0002% (w/v) Tween 20 in PBS, each well was incubated with 100 μl of horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson Immunochemicals) diluted 1/50000 in 5% (w/v) defatted milk in PBS and agitated at 37 °C for 2 h. The plate was then washed five times with 0.0002% (w/v) Tween 20 in PBS and developed with OPD and absorbance was read after 5–10 min at 495 nm.

Clone. Expression vector pM9PEX was introduced into QM7 cells by liposome-medium transfection. Two days after transfection, the cells were diluted to selection density (10^5/10-cm dishes) and selected in the growth medium supplemented with 400 μg/ml G418 (Gibco-BRL). The medium was refreshed every 3–5 days and resistant clones were identified in 2 weeks. Seven representative clones were expanded and screened for PEX production by Western blot using anti-c-myc antibody.

Protein extraction and purification. Clone 6 was subcultured in growth medium and then switched to serum-free medium containing 3 μg/ml insulin. After the cells were confluent, the medium was refreshed every 2 days for 6 days and the medium were collected and then purified sepa-

Table 1
The sequences of oligonucleotide primers used to construct expression plasmids

Synthetic sequence	Oligo name	Oligonucleotide sequence
IgG κ chain signal sequence	A	5′-AGC TTA TGA GCG TGC CGA CAC AGG TCC TGG GTT TGC TGC TGC TAT GGC TTA CAG ATG CGA GAT GTG ATA TCG-3′
	B	5′-GAT CCG ATA TCA CAT CTC GCA TCT GTA AGC CAT AGC AGC AGC AAA CCC AGG ACC TGT GTC GGC ACG CTC ATA-3′
MMP9 signal sequence	C	5′-AGC TTA TGA GCC TCT GGC AGC CCC TGG TCC TGG TGC TCC TGG TGC TGG GCT GCT GCT TTG CTG CCC CCA G-3′
	D	5′-GAT CCT GGG GGC AGC AAA GCA GCA GCC CAG CAC CAG GAG CAC CAG GAC CAG GGG CTG CCA GAG GCT CAT A-3′

rately. Nickel–nitrilotriacetic acid (Ni–NTA) affinity chromatography purification was performed using 500 μl Pro-Bond Ni^{2+} resin (Qiagen) washed with 30 ml ddH_2O and with 50 ml washing buffer (50 mM NaH_2PO_4-pH 8.0; 150 mM NaCl). Medium harvested at 2d, 4d and 6d after switching to serum-free medium (100 ml each) were cleared of cell debris by centrifugation and then were added to the resin and incubated for 2 h, at room temperature, with shaking. The resin was washed with 50 ml washing buffer containing 10 mM imidazole. The fractions were collected and the purified recombinant protein was eluted at a concentration of 150 mM of imidazole in 1 ml elution buffer (50 mM NaH_2PO_4, pH 8.0; 150 mM NaCl). The protein concentration of the fractions was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA). The supernatant was analyzed by SDS–PAGE.

Chicken chorioallantoic membrane (CAM) assay. Angiogenesis assays were performed essentially as described with minor modifications [9]. Five millimeter of filter discs were placed on the CAMs of 10-day-old chick embryos. 150 ng recombinant PEX or 1× PBS were added to the filter disc for 3 consecutive days. Then the CAM tissue associated with the filter disc was removed and photographed at 10× on an Olympus stereomicroscope.

Results

Evaluation of serum-free medium for myoblast differentiation

To determine whether the insulin-containing serum-free medium for myogenesis resulted in any differences from conventional differentiation medium, a time course of cell fusion was determined for QM7 cultures following switch to the two kinds of differentiation medium, and at various intervals the morphology was examined by Giemsa staining. QM7 cells maintained in growth medium consisted of a population of rapidly dividing mononucleated cells. Upon switch to differentiation medium, cells ceased dividing and fused to form elongated multinucleated myotubes. In both differentiation medium, the myoblasts started to fuse at 2d, formed myotubes containing several nuclei at 4d, and highly elongated multinucleated myotubes were formed at 6d (Fig. 1). No significant differences in the timing and degree of myogenesis were observed between insulin-containing and serum-containing culture.

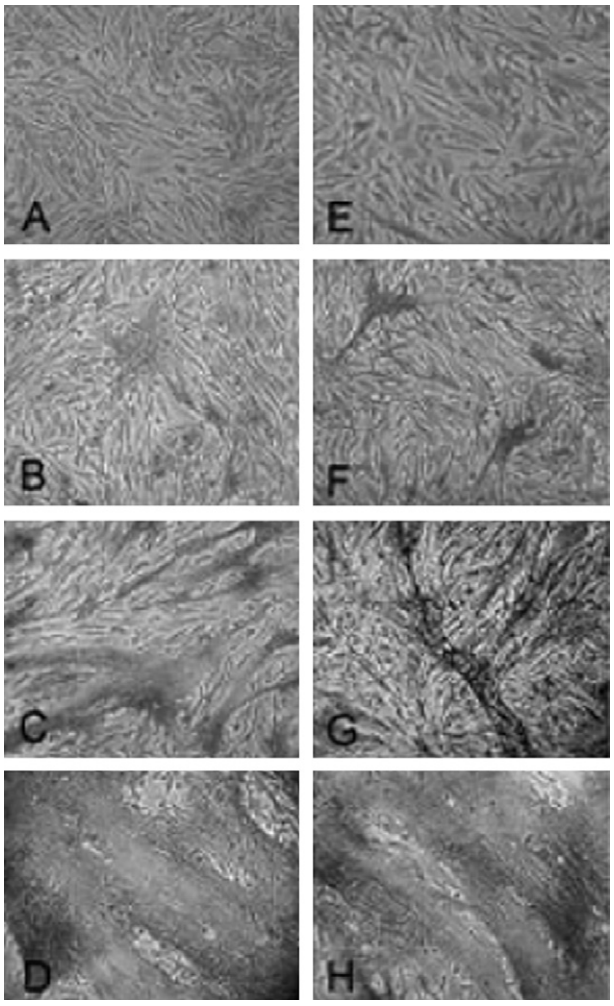

Fig. 1. Light microscopic examination of QM7 myoblasts during differentiation in various differentiation medium. 3×10^5 QM7 myoblasts were inoculated in 35 mm culture plates with growth medium, when cells were confluent, the medium was changed to 199 plus 0.5% fetal bovine sera (A–D) or serum-free 199 plus 3 μg/ml insulin (E–H) for induction of differentiation. The degree of myogenesis was morphologically assessed at 1d, 2d, 4d and 6d by Giemsa staining. Several large myotubes were observed in (C), (D), (G) and (H). (A) and (E), 1d; (B) and (F), 2d; (C) and (G), 4d; (D) and (H), 6d following switch to differentiation medium.

Effect of various signal peptide on the secretion of PEX

To test whether the three genetic fusion constructs could express PEX in QM7 cells, and if so, whether the mRNA expression levels differ among the various DNA constructs, the expression levels of PEX were measured at 6 h after transfection. The presence of mRNA transcripts of PEX was observed in all the DNA constructs. Moreover, semi-quantitative reverse transcription (RT-PCR) results show that there were no significant differences in the expression levels of PEX among the constructs at 6 h after transfection (Fig. 2A).

Though there was no difference in the expression levels of PEX at an early time point after transfection, the presence of a signal peptide sequence in the expression vector affected the trafficking of expressed proteins in the cells. Western-blotting of PEX protein from cell lysates and culture medium were measured at 24 h after transfection, resulting in the detection of proteins with predicted molecular mass. The highest amounts of PEX in culture medium and the lowest levels of PEX in the cytoplasm were obtained when pM9PEX was tested (Fig. 2B). In the case of pIgPEX and prGHPEX, equivalent amounts of intracellular and extracellular PEX were found.

PEX protein from cell pellets and culture medium were detected and quantified by ELISA at 48 h after transfection. As expected, the cells transfected with pM9PEX showed higher amounts of extracellular PEX and lower amount of intracellular PEX than that of pIgPEX and prGHPEX. The estimated yield for pM9PEX is about 2.4 mg/L (Fig. 2C).

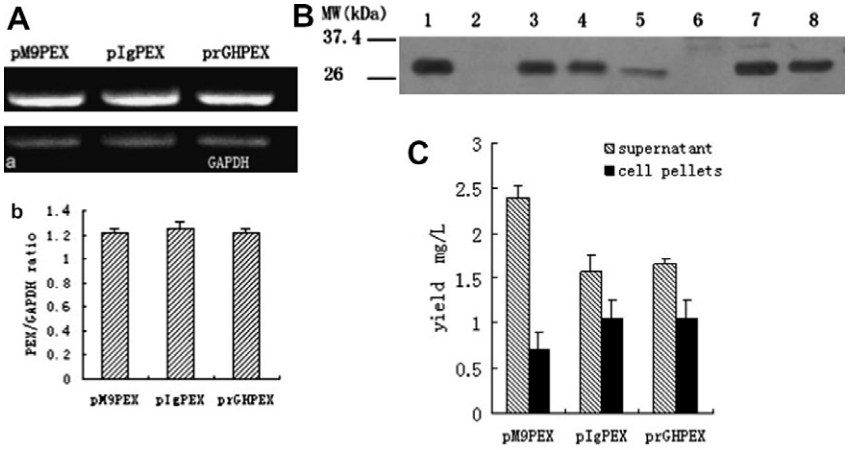

Fig. 2. Comparison of the secretion efficiency of various signal peptides. (A) mRNA expression levels of PEX in QM7 cells. (a) QM7 cells were transfected with various DNA constructs. At 6 h after transfection, the mRNA expression of PEX in the cell pellets was measured by reverse transcription–polymerase chain reaction. The cDNA samples were amplified by using specific primers of PEX and GAPDH. (b) The density ratios of PEX /GAPDH were determined for each DNA construct. The ratio of PEX/GAPDH was obtained by dividing the cDNA band density of PEX with that of the corresponding GAPDH. (B) Western blot analysis of PEX protein. QM7 cells were transiently transfected with 2 μg various expression constructs. At 24 h post-transfection, supernatant and cell lysates were subjected to Western blot analysis. Lane 1, pM9PEX supernatant; lane 2, pcDNA3 supernatant; lane 3, pIgPEX supernatant; lane 4, prGHPEX supernatant; lane 5, pM9PEX cell lysates; lane 6, pcDNA3 cell lysates; lane 7, pIgPEX cell lysates; lane 8, prGHPEX cell lysates, probed with monoclonal anti-c-myc antibody 9E10. Molecular mass standards (in kDa) are indicated on the left. (C) Detection the yield of PEX in QM-7 cells at 48 h after transient transfection with PEX expression vectors pM9PEX, pIgPEX, and prGHPEX.

Clone

QM7 cells were transfected with pM9PEX plasmid and stably transfected cells were cloned and selected for expression of recombinant PEX by Western blot using monoclonal anti-c-myc antibody. Among the positive clones, clone 6 exhibited a higher level of PEX comparison to other clones (Fig. 3A). Thus, it was chosen for further analysis.

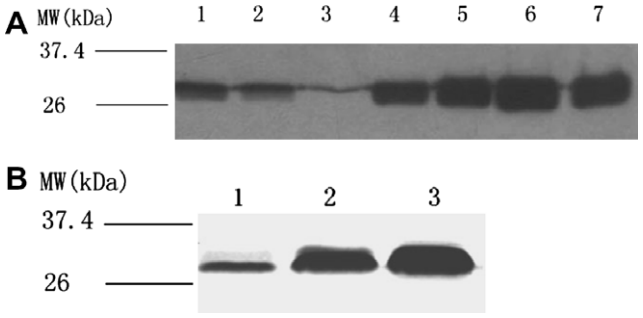

Fig. 3. Analysis of PEX protein in stable transfectants. (A) Screening of stable transfectants expressing PEX. QM7 cells were transfected with pM9PEX constructs and selected under G418 medium. The surviving colonies were cloned and expanded in 24-well plates. Upon confluency, cells were washed twice with PBS and replenished with 3 μg/ml insulin-containing medium. Twenty-four hours later, the conditioned medium were recovered and analyzed by Western blot without concentration as described. Monoclonal anti-c-myc antibody was used for the detection of PEX. Clones 1–7 (lanes 1–7) were shown here as representatives. (B) Ni^{2+} affinity purification of recombinant PEX from Clone 6. Clone 6 was subcultured in growth medium and then switched to 3 μg/ml insulin-containing medium. The serum-free medium was harvested at 2, 4 and 6 day following switch and PEX proteins were extracted by the Ni^{2+} affinity purification method. Purified product from medium harvested at 2d (lane 1), 4d (lane 2) and 6d (lane 3) after switching to insulin-containing medium were analyzed by SDS–PAGE/Coomassie staining.

Subculturing of clone 6 demonstrated that its level of expression for PEX was stable in subsequent passages.

Purification of histidine-tagged PEX from QM7 cells

To facilitate purification of recombinant PEX, the constructs were made containing a 6× histidine tag at the carboxyl terminus of the protein. Clone 6 was subcultured in growth medium and then switched to serum-free medium. The serum-free medium was harvested at 2, 4 and 6d following switch and PEX proteins were extracted by the Ni^{2+} affinity purification method. The SDS–PAGE analysis showed that the level of purified product from serum-free medium was elevated time dependently (Fig. 3B). The highest yield was exhibited at 6d when QM7 cells differentiated into large myotube and the estimated concentration is about 5 mg/L.

Antiangiogenic effect of PEX on the CAM

PEX blocks MMP-2 binding to αvβ3, resulting in disruption of angiogenesis. To determine if PEX expressed in QM7 cells is bioactive, angiogenesis assays were performed on the CAMs of chick 10-day old embryos. 150 ng of purified PEX protein applied in the center of a fiberglass filter paper on the surface of the CAM showed a clear antiangiogenic effect. Capillary growth was blocked under the filter paper. PBS alone did not interfere with CAM vasculature (Fig. 4).

Discussion

Modulation of the balance between pro- and antiangiogenic factors holds great promise for the treatment of a

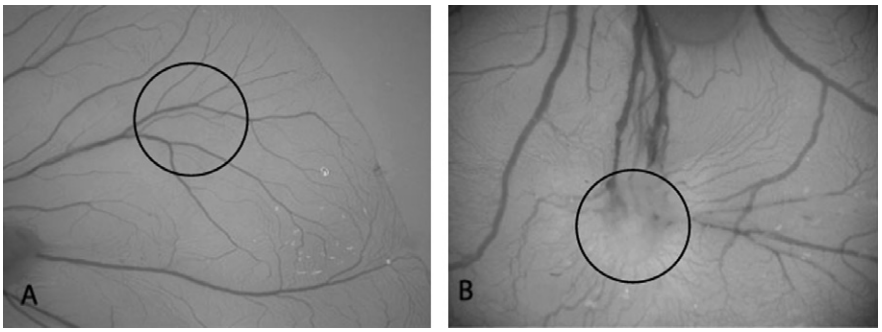

Fig. 4. PEX blocks angiogenesis in vivo. 1× PBS (A) or 150 ng recombinant PEX (B) was added to the filter disc CAMs of 10-day-old chick embryos for 3 consecutive days. Then the CAM tissue associated with the filter disc was removed and photographed at 10× on an Olympus stereomicroscope.

broad spectrum of human disease ranging from ischemic heart disease to cancer [10]. It has been shown that purified PEX, a noncatalytic fragment of MMP-2, prevents the binding of MMP-2 to integrin αvβ3, thereby disrupting angiogenesis [11]. Recent studys show that PEX inhibit glioma angiogenesis, cell proliferation, and migration [12,13], which makes PEX a powerful candidate for the treatment of malignant gliomas and possibly other human malignancies [14]. Overproduction of recombinant PEX protein is an important step toward the characterization of its structure-function relationships and is necessary to provide therapeutic quantities of recombinant PEX drugs. Due to the uncertainties in folding and modification associated with recombinant proteins produced in bacteria, it is often desirable to express them in eukaryotic cells. To achieve high-level expression in eukaryotic cells, the choice of cell line as well as the expression vector is critical.

The QM cell lines were derived from the preexisting quail fibrosarcoma cell line QT6, one of a series of cell lines cultured from methycholanthrene-induced pectoralis fibrosarcomas of Japanese quail *Coturnix japonica.* In most respects, QM cells closely resemble their mammalian counterparts. We discovered that QM7 cells are very potent in taking up DNA during transfection and the introduced genes are not constantly lost following mitosis. Differentiation of QM7 is serum dependent; following removal of serum, cells withdraw from the cell cycle and fuse into myotubes. Furthermore, myotubes can maintain survival for a long time and synthesize proteins in low-serum medium. The commercially available pcDNA3.0 vector carries the CMV promoter, which drives the expression of foreign genes, and a neomycin marker as a drug-resistance gene. As demonstrated below, the combination of pcDNA3.0-based expression vector and QM7 cells may prove to be a novel eukaryotic expression system.

In most of the previous reports concerning myoblast differentiation, QM7 cells were inoculated in medium containing 10% FBS and then switched to a fresh medium supplemented with a low concentration of serum. In those cultures, contamination by serum components could not be eliminated. As many recombinant proteins used for therapeutic and structural studies are naturally secreted, a serum-free culture medium that allows their efficient expression and purification is required. As insulin is reported to play an important role in the regulation of myoblast differentiation [15], serum-free cultures with 3 μg/ml insulin were prepared. Morphological comparison of this serum-free culture with conventional 0.5% FBS-containing culture revealed that there were no significant differences in the timing and degree of myogenesis between them. The result indicates that QM7 cells could differentiate well in serum-free medium. It is easy to purify secreted recombinant proteins from serum-free medium and ensure suitability for biotechnology medicine.

Efficient protein secretion is very important in biotechnology as it provides active and stable proteins. Most secretary proteins are synthesized in a precursor form containing a signal or leader sequence. This sequence contains 15–30 amino acids, which are involved in the beginning of the secretion pathway. Since the signal peptide is eventually removed during secretion, the replacement of native signal peptides by a strong foreign is not likely change the mature protein structure. Actually, protein signal peptide has become a crucial tool for researchers to have the recombinant protein secreted into the culture medium, by adding a specific signal peptide to the desired proteins [16]. In our primary research, MMP9 was expressed in QM7 cells at a level high enough to be detectable in crude supernatants without concentration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis stained with Coomassie brilliant blue R-250. The MMP9 signal peptide may enhance the secretion of the heterologous protein expressed in QM7 cells. In this study, three expression vectors containing PEX and three different signal peptides (the rat growth hormone, the Ig κ-chain and the MMP9 signal peptide) were constructed and the secretion efficiencies were compared. Given the similar mRNA expression levels of PEX derived from various DNA constructs in the cell pellets at 6 h after transfection, it is unlikely that pM9PEX could be less efficiently transcribed to PEX than pIgPEX and prGHPEX. Western-blotting of PEX from medium culture of pM9PEX showed a higher amount of extracellular PEX and lower amount of intracellular PEX than the other two constructs, which supported

the efficiency of MMP9 signal sequence in promoting PEX secretion. The ELISA study also showed that the highest amounts of extracellular PEX and lowest amount of intracellular PEX were detected in pM9PEX, which confirmed that MMP9 signal peptide is more effective than rGH and Ig light chain signal peptide.

To produce enough PEX in QM7 cells for analysis, stable transfectants were screened in 400 μg/ml G418. Clone 6 exhibited a higher level of PEX expression in comparison to other clones and it was chosen for further analysis. Subculturing of clone 6 demonstrated that its level of expression of PEX was stable in subsequent passages. The PEX proteins were extracted from serum-free medium of clone 6 by Ni^{2+} affinity purification method. The SDS–PAGE analysis showed that the level of purified product from serum-free medium was elevated time dependently. The increment of the yield of PEX was accompanied by the degree of QM7 cells differentiation. The highest yield was exhibited at 6d when QM7 cells differentiated into large myotubes. Myotubes can survive for a long time in this serum-free medium and maintain producing recombinant proteins. This advantage can be taken to get a large scale of recombinant proteins without contamination of serum. About 0.5 mg PEX was purified from 100 ml medium at 6d.

To detect the bioactivity of PEX purified from medium, its antiangiogenic effects on the CAM were assayed. One hundred and fifty nanograms of purified PEX protein blocked capillary growth of CAM. The data indicated that PEX protein was modified thoroughly and secreted successfully into the culture medium with bioactivity.

In summary, we have established a novel skeletal muscle expression system. A type of serum-free medium was found to be suitable for QM7 cell differentiation and recombinant protein purification. MMP9 signal peptide proved to be efficient on directing recombinant protein secretion in QM7cells. PEX protein was purified successfully from culture medium with bioactivity.

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