ELSEVIER

Cell Biology International 32 (2008) 739–747

Cell Biology International

www.elsevier.com/locate/cellbi

Expression of recombinant anticoagulant hirudin in the differentiated cultures of the porcine mammary epithelial cell line SI-PMEC

Y.L. Sun [a], Y.C. Chou [a], T.C. Kuan [b], C.F. Tu [a], C.S. Lin [b,*]

[a] *Division of Biotechnology, Animal Technology Institute, Taiwan, P.O. Box 23, Chunan 35099, Miaoli, Taiwan*
[b] *Department of Biological Science and Technology, National Chiao Tung University, No. 75 Po-Ai Street, Hsinchu 30068, Taiwan*

Received 7 September 2007; revised 22 October 2007; accepted 25 February 2008

Abstract

To express recombinant proteins in the spontaneously immortalized porcine mammary epithelial cell line (SI-PMEC) currently established in our laboratory, a chemically synthesized DNA fragment encoding the anticoagulant hirudin was used to construct a mammalian expression vector under the control of the goat β-casein regulatory sequence. The vector, named pGB562/Hi, was transfected into the SI-PMEC cells to yield pGB562/Hi/SI-PMEC. The pGB562/Hi/SI-PMEC cells expressed recombinant hirudin only when they were differentiated into functional structures by growth on a Matrigel-coated petri dish supplemented with the lactogenic hormone prolactin. The differentiated pGB562/Hi/SI-PMEC cells produced about 0.5–0.6 μg of recombinant hirudin/mg of total cellular protein. These results suggest that the established SI-PMEC cells have pharmaceutical potential to inducibly express bioactive heterogeneous proteins.

Keywords: Recombinant protein; Mammary gland; Epithelial cell; SI-PMEC; Hirudin

1. Introduction

We have established a spontaneously immortalized porcine mammary epithelial cell line, SI-PMEC, from the mammary gland of a lactating sow and maintained it in culture long-term by continuous subculturing (Sun et al., 2005, 2006). SI-PMEC cells can differentiate into functional structures and will express and secrete milk proteins when cultured on Matrigel-coated petri dishes supplemented with lactogenic hormone (Sun et al., 2006). It is unclear, however, whether the SI-PMEC cells can potentially express exogenous recombinant genes and produce bioactive proteins, as it had not been assessed prior to this study.

The anti-thrombotic polypeptide hirudin, which is isolated from the salivary glands of *Hirudo medicinalis*, contains 64–66 amino acids (Markwardt, 1970; Harvey et al., 1986). Hirudin binds specifically to thrombin, a protein involved in the blood clotting cascade, and thereby inhibits coagulation (Dodt et al., 1984; Seemüller et al., 1986). Therefore, recombinant hirudin is useful for treating diseases related to the coagulation activity of thrombin or for preventing, alleviating or ameliorating symptoms of these diseases, which include acute coronary syndromes (Matheson and Goa, 2000; Weitz and Bates, 2003; Greinacher, 2004).

In the present study, we sought to determine whether a synthetic hirudin gene could be transfected into the porcine mammary epithelial cell line, SI-PMEC, and subsequently induced to generate bioactive recombinant hirudin. We also characterized the inducing factors and expression potential of the bioactive heterogeneous protein in the SI-PMEC culture system.

Abbreviations: SI-PMEC, spontaneously immortalized porcine mammary epithelial cell line; RT-PCR, reverse-transcription polymerase chain reaction; GB562, a DNA fragment (5.62 kb) containing the 5′ flanking sequence and intron 1 of the goat β-casein gene; Hi, Hirudin; EGFP, enhanced green fluorescent protein, a reporter used in gene transfection; pGB562/GFP, an expression vector containing an EGFP encoding gene fragment which is controlled under the sequence of GB562; pGB562/Hi, an expression vector containing a hirudin encoding gene fragment which is controlled under the sequence of GB562; pGB562/GFP/SI-PMEC, the SI-PMEC cells transfected with the expression vector of pGB562/GFP; pGB562/Hi/SI-PMEC, the SI-PMEC cells transfected with the expression vector of pGB562/Hi.

* Corresponding author. Tel.: +886 3 5131338; fax: +886 3 5729288.
E-mail address: lincs@mail.nctu.edu.tw (C.S. Lin).

1065-6995/$ - see front matter © 2008 International Federation for Cell Biology. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.cellbi.2008.02.004

2. Materials and methods

2.1. Cell culture

The culture of SI-PMEC cells was performed as in our previously reported protocol (Sun et al., 2006). For cell growth and proliferation, the cells were cultured with the basal medium, DMEM/F12 (GIBCO, Carlsbad, CA, USA) containing 10% fetal calf serum (batch number #716543; Biological Industries, Kibbutz Beit Haemek, IL, USA), insulin (10 μg/ml; Sigma, Canton, MA, USA), and hydrocortisone (1 μg/ml; Sigma), in a humidified atmosphere containing 5% CO_2 at 37 °C. The fetal calf serum was pretreated by heat inactivation at 56 °C for 30 min before use. For cell differentiation with secretory function, the cells were cultured onto petri dishes coated with BD Matrigel™ (BD Biosciences Clontech, Franklin Lakes, NJ, USA) and the basal medium was supplied with prolactin (5 μg/ml; Sigma).

2.2. Synthesis of the DNA fragment encoding hirudin

The DNA fragment encoding hirudin was synthesized according to the method reported by Kochanowski et al. (2006) with slight modifications. Based on the sequence of the hirudin gene disclosed in GenBank accession number M12693, two oligonucleotides, Hi-1 (123-mer) and Hi-2 (128-mer) were designed to build the gene encoding hirudin, wherein 15-mer at the 3′-end of both strands are complementary to each other. In addition, two primers, Hi-PCR-F (33-mer) and Hi-PCR-R (32-mer), were designed according to the 5′-terminal sequences of the above Hi-1 and Hi-2 oligonucleotides and enabled to clone the gene by restriction endonucleases, SalI and NotI (Table 1 and Fig. 1A). All of the oligonucleotides were synthesized by Applied Biosystems (Foster City, CA, USA).

A reaction mixture containing 8 μl of each the oligonucleotides Hi-1 and Hi-2 (10 μM of each), 10 μl dNTPs (2 mM), 0.5 U Taq DNA polymerase (HT Biotechnology, Cambridge, UK), 5 μl of 10× PCR buffer (15 mM $MgCl_2$, 500 mM KCl, 1% Triton X-100, 0.1% gelatin and 100 mM Tris–HCl, pH 7.9), and 18 μl H_2O, was incubated at 70 °C for 30 min. The primers Hi-PCR-F and Hi-PCR-R were used to amplify the gene encoding hirudin as well. The reaction mixture was heated at 94 °C for 5 min before entering the PCR cycles in an Applied Biosystems DNA thermal cycler (Applied Biosystems). The reaction conditions were 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. After a total of 36 cycles, the mixture was then subjected to 72 °C for 10 min to complete the DNA extension.

2.3. Construction of the hirudin expression vector

The construction flowchart of the hirudin expression vector is shown in Fig. 1A. The vector of pGB562/GFP (10.40 kb), a mammary gland-specific expression vector which contains 5.62 kb of the 5′ flanking sequence and intron 1 of goat β-casein gene linked to a gene fragment encoding enhanced green fluorescent protein (EGFP) (Wu et al., 2003), was used in this study as a control of DNA transfection and expression as well as also providing the regulatory region of goat β-casein gene to transcribe EGFP or hirudin mRNA. The pGB562/GFP vector was treated with SalI and NotI to remove the EGFP DNA fragment and then was ligated with the synthesized hirudin gene fragment which was also digested with SalI and NotI to obtain a hirudin expression vector, pGB562/Hi, which can specifically express hirudin in differentiated mammary epithelial cells.

2.4. Gene transfection of SI-PMEC

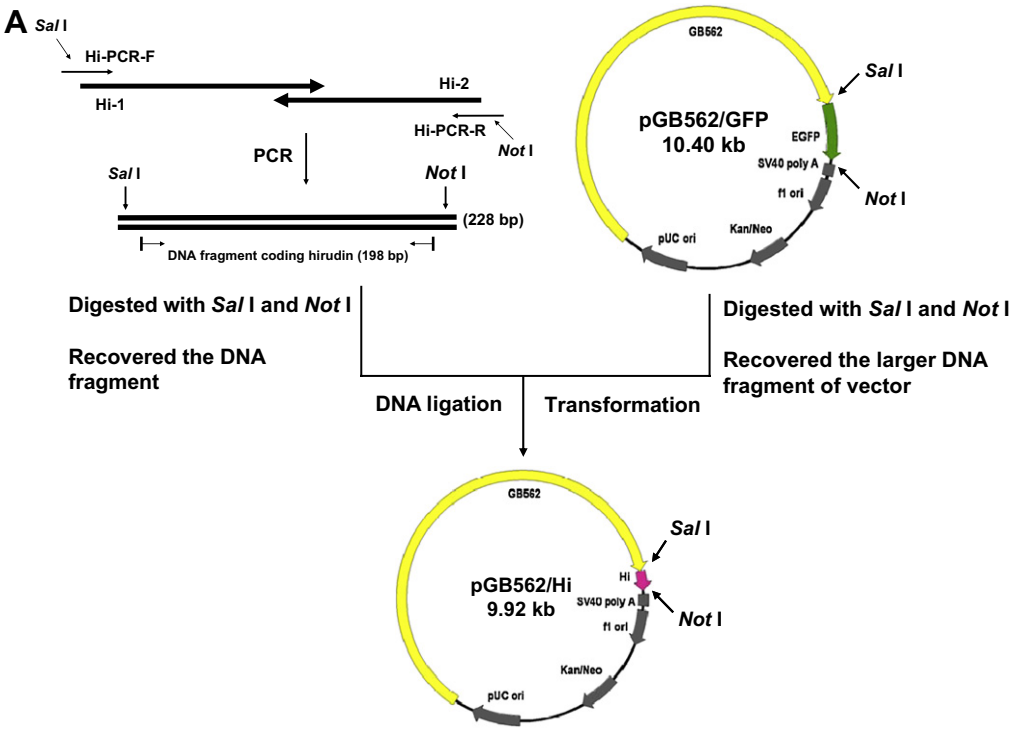
The SI-PMEC cells were transfected with pEGFP-N1 (a vector constructed with a CMV promoter followed by the EGFP gene) (BD Biosciences Clontech), pGB562/Hi or pGB562/GFP by electroporation. After mixing the plasmid DNA (2–4 μg) with SI-PEMC cells (1×10^6 cells in 0.4 ml), the mixture was put into an electroporation cuvette 0.4 cm in width and treated by an electroporator (BTX 830; Gentronics, San Diego, CA, USA) under the following conditions: 200–500 mV/cm at 50 mV intervals, 99 μs, and pulsed 4 times. The treated cells were moved into a 60 mm petri dish and cultured with basal medium at 37 °C in a 5% CO_2 atmosphere for 4 h, and then replaced with fresh medium. After 24 h culturing, the expression of GFP in the DNA transfected SI-PMEC cells was observed using a fluorescence microscope (Axiovert 135; Carl Zeiss, Gottingen, Germany) with fluorescein isothiocyanate (FITC) optics.

Geneticin (G418; 500 μg/ml; Sigma) was also used for selection of transfected SI-PMEC cells, and the culture medium

Table 1
Sequences of oligonucleotides used to synthesis of full-length DNA fragment encoding hirudin

Oligonucleotide	Sequence (5′ → 3′)	Number of mer
Template oligonucleotides		
Hi-1	gatccttt atg gtt gtt tac act gac tgc act gaa tcc ggt cag aac ctg tgc ctg tgc gaa ggc tct aac gtt tgc ggc cag ggc aac aaa tgc atc ctg ggc tct **gat** ggc gaa aaa aat c	123
Hi-2	ggccgcccta tta ttg cag gta ttc ttc cgg gat ttc ttc aaa gtc gcc gtc gtt gtg aga ctg cgg ttt cgg agt acc ttc gcc agt aac gca ttg att ttt ttc gcc **atc** aga gcc cag gat gca t	128
Primer oligonucleotides		
Hi-PCR-F	tcg**gtcgac**ttt atg gtt gtt tac act gac tgc (SalI)	33
Hi-PCR-R	cat**gcggccgc**ccta tta ttg cag gta ttc tt (NotI)	32

The start codon ATG designed in the synthesized hirudin gene is indicated with bold letters in the Hi-1 and Hi-2 sequences. The arranged cutting sites by restriction enzyme SalI and NotI are indicated with bold letters in the Hi-PCR-F and Hi-PCR-R sequences, respectively.



B

```
        Sal I
5'-tcggtcgacttt ATG GTT GTT TAC ACT GAC TGC ACT GAA TCC GGT CAG
                Met Val Val Tyr Thr Asp Cys Thr Glu Ser Gly Gln

AAC CTG TGC CTG TGC GAA GGC TCT AAC GTT TGC GGC CAG GGC AAC AAA
Asn Leu Cys Leu Cys Glu Gly Ser Asn Val Cys Gly Gln Gly Asn Lys

TGC ATC CTG GGC TCT AGA GGC GAA AAA AAT CAA TGC GTT ACT GGC GAA
Cys Ile Leu Gly Ser Asp Gly Glu Lys Asn Gln Cys Val Thr Gly Glu

GGT ACT CCC AAA CCG CAG TCT CAC AAC GAC GGC GAC TTT GAA GAA ATC
Gly Thr Pro Lys Pro Gln Ser His Asn Asp Gly Asp Phe Glu Glu Ile

CCG GAA GAA TAC CTG CAA TAA tagggcggccgcatg-3'
Pro Glu Glu Tyr Leu Gln Stop
                          Not I
```

Fig. 1. Schematic presentation of the procedure to synthesize the DNA fragment encoding hirudin and to construct the expression vector, pGB562/Hi. (A) The synthesized Hi-1 and Hi-2 were used as templates and the primer pair, Hi-PCR-F and Hi-PCR-R, was used to amplify the DNA fragment (228 bp) by PCR. Restriction enzyme SalI and NotI sites were designed at the 5′ and 3′ termini in the DNA fragment, respectively. The amplified 228 bp fragment contained a 198 bp hirudin gene. The construction and features of the vector are detailed in the Section 2. Abbreviations: pGB562, a DNA fragment containing the 5′ flanking sequence and intron 1 of goat β-casein gene; Ap, ampicillin resistance gene; Kan/Neo, kanamycin and neomycin resistance gene; Hi, hirudin variant I coding gene; Ori, replication origin from pUC plasmid. (B) The nucleotide sequences of the synthesized DNA fragment encoding hirudin. The nucleotide sequences listed are under the predicted coding arrangement. The amino acid sequence deduced from the DNA sequence was shown under the corresponding codons.

was replaced irregularly thereafter. After one successive generation, SI-PMEC cells that stably contained pGB562/Hi or pGB562/GFP (pGB562/Hi/SI-PMEC or pGB562/GFP/SI-PMEC) were obtained.

2.5. Determination of β-casein and hirudin transcripts

For determining β-casein, a major milk protein, and hirudin gene expression induced by cultured substrata and lactogenic hormone, the pGB562/Hi/SI-PMEC cells cultured on a Matrigel-coated petri dish in basal medium containing prolactin (5 μg/ml; Sigma) were used to prepare total cellular RNA for reverse transcription-polymerase chain reaction (RT-PCR). The PCR primers for amplification of β-casein and hirudin transcripts were as follows:

(1) Porcine β-casein (GenBank accession no. X54974)
β-ca-F (21-mer) 5′-CCA AAG CTA AGG AGA CCA TTG-3′
β-ca-R (19-mer) 5′-CAA CTG GTT GAG GCA CAG G-3′
(2) Hirudin (GenBank accession no. M12693)
Hi-F (21-mer) 5′-TAC ACT GAC TGC ACT GAA TCC -3′
Hi-R (18-mer) 5′-TTG CAG GTA TTC TTC CGG-3′

Total cellular RNA preparation and RT-PCR were performed as previously described (Lin and Hsu, 2005). Total

RNA was extracted from SI-PMEC (1×10^6 cells) using Trizol (GIBCO) in accordance with the manufacturer's instructions. Five micrograms of the DNase I (Promega, Madison, WI, USA) treated total RNA were reverse-transcribed using 2.5 μM oligo-dT primers (Promega), 1 mM of each dNTP (Promega), 20 U ribonuclease inhibitor (HT Biotechnology) and 5 U reverse transcriptase (HT Biotechnology) in RT buffer (25 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM DTT and 5 mM $MgCl_2$) in a total volume of 20 μl at 39 °C for 60 min. For each PCR reaction, 3 μl of RT product were added to a final volume of 50 μl containing 10 mM Tris–HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% Triton X-100, 0.001% gelatin, 200 μM of each dNTP, 0.5 U Taq DNA polymerase (HT Biotechnology) and 0.2 μM of each primer pair. For the gene expression assay, PCR was carried out for 28 cycles with 30 s denaturation at 94 °C, 45 s annealing at 55 °C, and 45 s extension at 72 °C.

2.6. Western blot assay

Cultured SI-PMEC cells were recovered to prepare homogeneous extract by sonication and centrifugation. The total amount of protein in homogeneous extract was measured by the Bradford dye binding assay (BioRad Laboratories, Hercules, CA, USA) and bovine serum albumin as the standard.

The Western blot was performed as in our previous report (Pan et al., 2007). Equal amounts of protein and the commercial hirudin standard (Recombinant hirudin #377853, vial of 200 μg, ca. 2000 ATU; Calbiochem, EMD Biosciences, Darmstadt, Germany) were separated by SDS-polyacrylamide gel electrophoresis (NuPAGE 4–12% Bis-Tris Gel, Invitrogen, Carlsbad, CA, USA). After electrophoresis, the proteins were transferred to Nitropure membrane (Micron Separations, Westborough, MA, USA), and then blotted with the mouse monoclonal hirudin antibody (diluted 1:2000; Abcam, Cambridge, UK) for 2 h. After washing with 1× D-PBS, the membrane was incubated with secondary antibody of anti-mouse IgG conjugated with alkaline phosphatase (AP) (diluted 1:2500; Sigma), and then developed with AP Immunoblot Assay Developer (BioRad Laboratories).

2.7. Immunocytochemistry

The SI-PMEC cells were plated in 6-well plates (1×10^5 cells/well) for 24 h and then fixed in 95% ethanol for 1 min. Twenty microliters of mouse monoclonal hirudin antibody (diluted 1:500) were applied per well and the plates were incubated at 37 °C for 1 h in a 100% humidity chamber. The plates were then washed (3×5 min) with D-PBS at room temperature and the cells were incubated with secondary antibody using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations. The incubation and washes were as described above. Finally, the cells were stained with a Vector TMB Substrate Kit (Vector Laboratories) and examined by light microscopy (Sun et al., 2006).

2.8. Assay of anti-coagulation activity of hirudin

The hirudin activity in the homogeneous extract was determined through anti-thrombin activity using a chromogenic substrate, Chromozyme TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide; Roche, Mannheim, Germany) (Sohn et al., 1991; Kim et al., 2001). The amidolytic cleavage of Chromozyme TH by thrombin (Sigma) was measured as the rate of increase in absorbance at 405 nm with a microtiter plate reader (Bio-Tek, Winooski, VT, USA). Thrombin (0.6 NIH unit/ml), diluted culture supernatant and Chromozyme TH (200 μM) were loaded into the 96-well assay plate simultaneously. The reaction was monitored every 30 s for 5 min. One anti-thrombin activity (ATU) was defined as the amount of hirudin able to completely inhibit one NIH unit of human thrombin at 37 °C. Commercial recombinant hirudin (Calbiochem) was used as a standard to estimate the hirudin concentration by the activity measurement (Sohn et al., 1991).

3. Results

3.1. Construction of the hirudin expression vector

Synthesis of the gene encoding hirudin was based on two designed oligonucleotides, Hi-1 and Hi-2, for which the codon sequences corresponded to the amino acid sequences found in natural hirudins, with a change to add an initiating ATG codon at the 5′-terminal of the open reading frame of hirudin (Fig. 1A). The size of the DNA fragment amplified by PCR using the Hi-PCR-F and Hi-PCR-R as primer pair and the hybrid DNA of Hi-1 and Hi-2 as template was 228 bp. The restriction enzyme-treated (SalI and NotI) synthesized DNA fragment (including an open reading frame covering 198 bp) was inserted into a vector with a 5′ flanking sequence and intron 1 of the goat β-casein gene as a regulatory sequence (5.62 kb) to yield the construct pGB562/Hi (9.92 kb) (Fig. 1A). The inserted hirudin gene was sequenced and was identical to the original design as shown in Fig. 1B.

3.2. Gene transfection and GFP expression in SI-PMEC cells

Plasmid pEGFP-N1 was used to transfect SI-PMEC cells to test the efficiency of gene transfection via electroporation and the effect on cell survival. Cell survival was generally decreased in the range from 95 to 24%, and the efficiency of transfection generally increased from 1.2 to 17.1% when the electroporation voltage was increased from 200 to 500 mV at 50 mV intervals. These efficiencies were lower than that of pEGFP-N1 transfected into primary epithelial cells of the porcine mammary gland (Sun et al., 2005). However, the population of EGFP-expressing SI-PMEC cells could optimally reach over 30% after one passage with G418 selection. This transfection efficiency was acceptable for further experiments involving pGB562/GFP and pGB562/Hi transfection and for determining the optimal conditions for inducible expression

of GFP and hirudin in the pGB562/GFP/SI-PMEC and pGB562/Hi/SI-PMEC cells, respectively.

3.3. Inducible GFP expression in pGB562/GFP/SI-PMEC cells

The SI-PMEC cells transfected with the mammary gland-specific expression vector pGB562/GFP (named pGB562/GFP/SI-PMEC cells) were used to test the potential to express recombinant protein in transfected SI-PMEC cells. The pGB562/GFP/SI-PMEC cells showed the characteristic cobblestone morphology of epithelial cells (Fig. 2A); the fluorescent signal of the culture could not be observed under the fluorescence microscope with FITC optics (Fig. 2B) when the cells were routinely cultured in basal medium on a petri dish. However, when the pGB562/GFP/SI-PMEC cells were cultured on a Matrigel-coated petri dish and the basal medium was supplemented with prolactin, the cells differentiated and formed a functional morphology; that is, the cells aggregated to form alveolar- and duct-like structures (Fig. 2C). With continued incubation of the cultures, the duct-like structures were not only maintained but continued to develop into a tubular network with lumens (data not shown). These differentiated pGB562/GFP/SI-PMEC cells produced strong GFP fluorescence (Fig. 2D). These results indicate that recombinant protein was inducible and could be produced in SI-PMEC after transfecting with a mammary gland-specific expression vector.

3.4. Expression of recombinant hirudin in pGB562/Hi/SI-PMEC cells

Hirudin expression in pGB562/Hi/SI-PMEC cells was detected by immunocytochemistry, Western blotting and RT-PCR. Immunocytochemistry showed very little hirudin expression when the cells were cultured in basal medium on petri dishes (Fig. 2E); however, hirudin was strongly expressed when pGB562/Hi/SI-PMEC cells were cultured on Matrigel-coated dishes supplemented with prolactin (Fig. 2F). The expression of recombinant hirudin in pGB562/Hi/SI-PMEC cells was confirmed by Western blotting (Fig. 3). Hirudin was detected in the differentiated pGB562/Hi/SI-PMEC cells and exhibited the same molecular mass in the electrophoretic SDS-polyacrylamide gel (18–20 kDa) as the commercial recombinant hirudin.

Furthermore, hirudin expression at the transcription level was detected by RT-PCR (Fig. 4). The transcription results are basically consistent with the translation results. In addition to morphology, expression of milk protein transcripts is also a significant marker to determine the status of SI-PMEC cell differentiation. Therefore, β-casein and hirudin transcripts were evaluated in this study. When the pGB562/Hi/SI-PMEC cells were grown on a plastic substratum, the β-casein transcript was detected at a very low level and hirudin was undetectable. In contrast, when the cells were cultured on a Matrigel-coated petri dish supplemented with 5 μg/ml prolactin, the β-casein and hirudin were strongly expressed (Fig. 4).

3.5. Bioactivity assay of hirudin expressed by pGB562/Hi/SI-PMEC cells

The anti-coagulation bioactivity of hirudin in homogenate of pGB562/Hi/SI-PMEC cells was assessed. The bioactivity of expressed recombinant hirudin was evaluated by determining absorption changes at 405 nm caused by inhibition of the thrombin reaction using Chromozyme TH. Commercial hirudin was used to establish a standard curve (from 0 to 60 ng/ml at 15 ng/ml intervals) (Fig. 5A). Based on the standard curve and the measured values, hirudin in the cells was quantified. As shown in Fig. 5B, activity equivalent to the background was detected in the homogenate prepared from undifferentiated pGB562/Hi/SI-PMEC cells. The results of artificially adding hirudin into the cell homogenate indicate that the detected hirudin activity would not be affected by the hirudin in the homogenate. These results indicate that this method could be used to determine hirudin production in SI-PMEC cell homogenate. The production of recombinant hirudin in the differentiated pGB562/Hi/SI-PMEC cells was estimated to be 0.5–0.6 μg/mg of total cellular protein (Fig. 5B).

The activity of hirudin in the milk stored at 4 °C in a refrigerator for 1 week was decreased by approximate 25% compared with that of the fresh milk sample containing recombinant hirudin. We proposed that the decreased activity of hirudin in the stored milk might due to degradation of the hirudin or conjugation with milk proteins during storage.

3.6. Effect of Matrigel and prolactin on recombinant protein expression

The effects of Matrigel and prolactin on recombinant hirudin expression in pGB562/Hi/SI-PMEC cells were determined. The pGB562/Hi/SI-PMEC cells were cultured in the basal medium without or with supplemental prolactin (5 μg/ml) and grown on petri dishes lacking or containing Matrigel coating. Recombinant hirudin production in the pGB562/Hi/SI-PMEC cultures was enhanced about 12- and 21-fold by prolactin and Matrigel, respectively (Fig. 5C). The recombinant hirudin production was enhanced 29-fold when the pGB562/Hi/SI-PMEC cells were cultured on a Matrigel-coated petri dish and supplemented with prolactin.

4. Discussion

This study describes the expression of a heterogeneous protein, hirudin, in the immortalized porcine mammary epithelial cell line, SI-PMEC. The hirudin gene was synthesized and used to construct a mammary expression vector controlled under the regulatory sequences of goat β-casein, a tissue- and stage-specific expression promoter (Wu et al., 2003; Rijnkels and Pieper, 1989). Thus, hirudin was expressed upon differentiation of hirudin gene-transfected SI-PMCE cells into functional structures induced by growth on a Matrigel-coated dish supplemented with prolactin.

β-Casein is the most abundant protein in the milk of goats and cows. Accordingly, it has been suggested that the β-casein

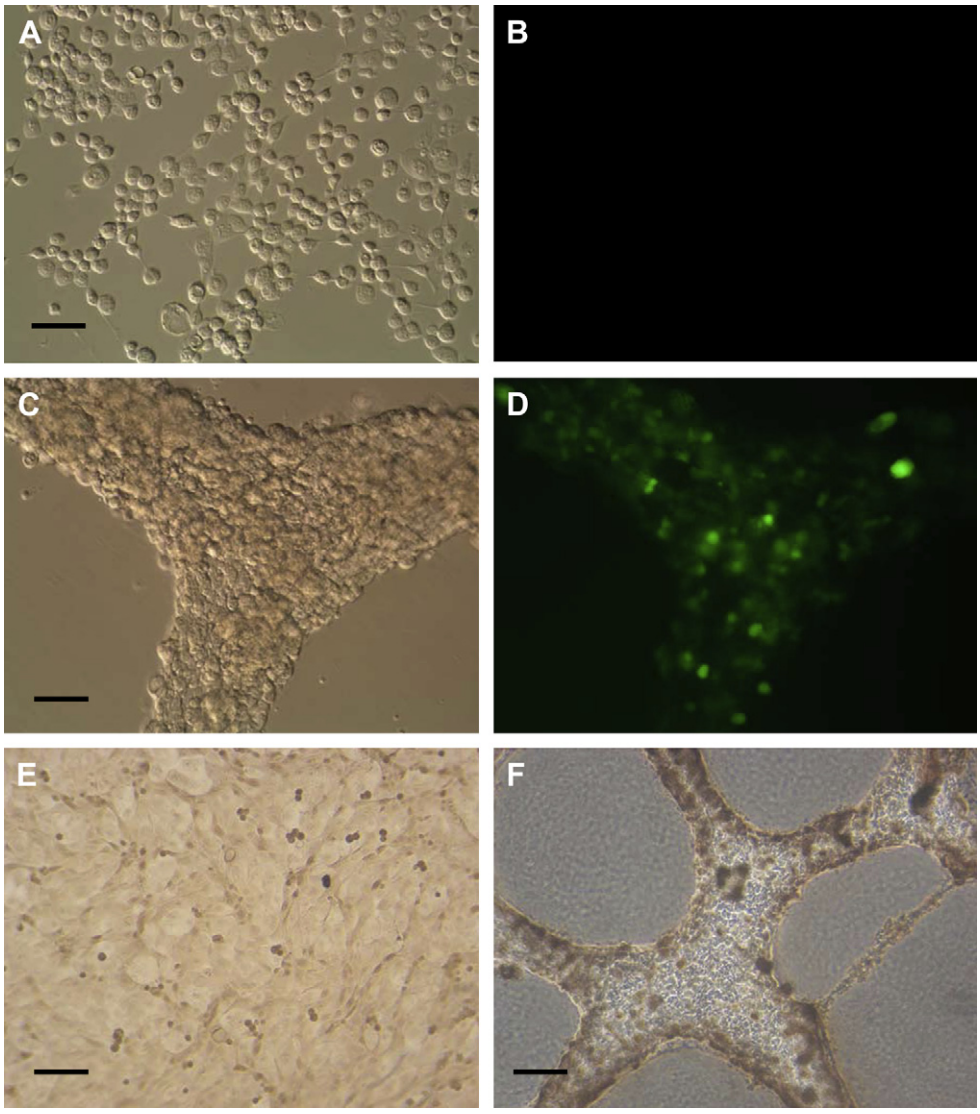


Fig. 2. Inducible targeting expression in SI-PMEC cells transfected with pGB562/GFP (pGB562/GFP/SI-PMEC) and pGB562/Hi (pGB562/Hi/SI-PMEC). (A) An image under bright field of pGB562/GFP/SI-PMEC cells cultured in basal medium. (B) The image under a fluorescent field, which is the same field shown in panel (A). (C) An image under a bright field of pGB562/GFP/SI-PMEC cells cultured on a Matrigel-coated petri dish and basal medium provided with prolactin. Morphological differentiation (duct-like structures developed into a tubular network with lumens) of pGB562/GFP/SI-PMEC cells cultured with Matrigel and supplemental prolactin was observed. (D) The image under the fluorescent field is the same field shown in panel (C). (E) The undifferentiated pGB562/Hi/SI-PMEC cells cultured in basal medium and treated with a mouse monoclonal antibody against hirudin. The cells produced no immunopositive signal. (F) pGB562/Hi/SI-PMEC cells cultured on a Matrigel-coated petri dish and basal medium supplemented with prolactin and treated with anti-hirudin monoclonal antibody. The many cells that formed duct-like structures developed into a tubular network with lumens and were immunopositive (deep brown color) for hirudin, indicating that the recombinant hirudin was expressed in these functional-structural pGB562/Hi/SI-PMEC cells. Bars indicate 200 μm.

gene is a powerful promoter that may be suitable for use in driving transgene expression in the mammary glands of transgenic animals (Roberts et al., 1992; Ebert et al., 1994). Several elements, including composite response element (CoRE), signal transducers and activators of transcription (STAT), CCAAT/enhancer binding protein (C/EBPs), glucocorticoid response element (GRE), pregnancy-specific mammary nuclear factor (PMF) and octamer-binding protein (Oct), within the promoter region of the gene were identified by motif assay or experimental study (Groner and Gouilleux, 1995; Gorodetsky and Bremel, 1998; Rosen et al., 1999). In addition to being in the regulatory region of the gene, intron 1 is also essential for the gene regulation. Kang et al. (1998) indicated that prolactin-inducible enhancer activity was localized in intron 1 of the bovine β-casein gene. In addition, several elements in intron 1 of the bovine β-casein gene interact cooperatively not only with each other, but also with its promoter for hormonal induction (Petitclerc et al., 1995). The present results suggest that the 6.2-kb regulative sequence of the goat β-casein gene is sufficient and effective for directing exogenous gene expression in the SI-PMECs. The results were confirmed by the report from Wu et al. (2003).

A number of immortal mammary epithelial cell lines of cow (German and Barash, 2002; Rose et al., 2002) have been established. The cell lines could be used to produce bioactive recombinant protein. Lazaris et al. (2002) used the

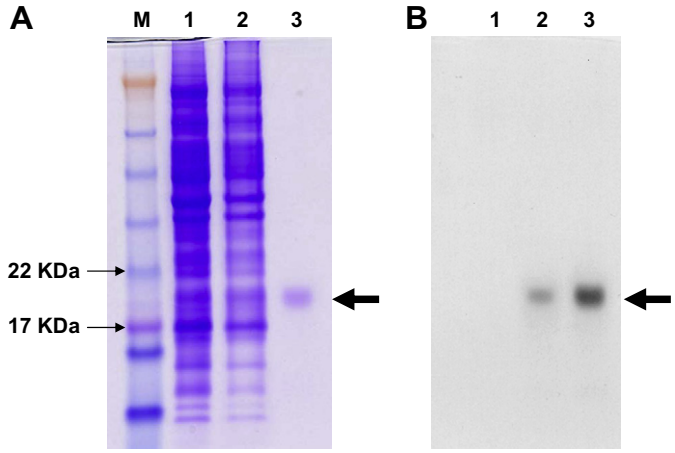


Fig. 3. Western blot analysis of recombinant hirudin expressed in pGB562/Hi/SI-PMEC cells. (A) The samples were subjected to 12% SDS-polyacrylamide gel electrophoresis under reducing conditions. (B) The parallel samples in (A) were separated on an SDS-polyacrylamide gel, transferred to a nylon membrane and then detected by enhanced chemiluminescence using a mouse monoclonal antibody against hirudin. M, a protein marker; molecular masses at 17 and 22 kDa are indicated; lane 1, the homogenate (20 μg) from pGB562/Hi/SI-PMEC cells cultured in basal medium and grown on a petri dish; lane 2, the homogenate (10 μg) from pGB562/Hi/SI-PMEC cells cultured on a Matrigel-coated petri dish and basal medium supplemented with 5 μg/ml prolactin; lane 3, 5 ng of commercial recombinant hirudin. In lanes 2 and 3, bands immunopositive for hirudin were detected. Hirudin is indicated by arrows.

immortalized bovine mammary epithelial MAC-T cells to produce soluble recombinant spider silk. Neumann et al. (2006) also used a similar system to produce recombinant equine prorelaxin in transfected MAC-T cells and this prorelaxin could induce a biological response in an *in vitro* bioassay.

Recombinant hirudins have been produced using a variety of expression systems, including yeast (*Saccharomyces cerevisiae* and *Pichia pastoris*) (Sohn et al., 1991; Kim et al., 2001; Zhou and Zhang, 2002; Shi et al., 2006), bacteria (*Escherichia coli*) (Kochanowski et al., 2006; Tan et al., 2002), and in mammalian cell lines (CHO and HBK) (Riesbeck et al., 1998; Guarna et al., 2000). In the present study, recombinant hirudin was expressed in an immortalized porcine mammary epithelial cell line, SI-PMEC. The SI-PMEC cells have all the machinery to properly fold a mammalian protein, which is important for biological activity.

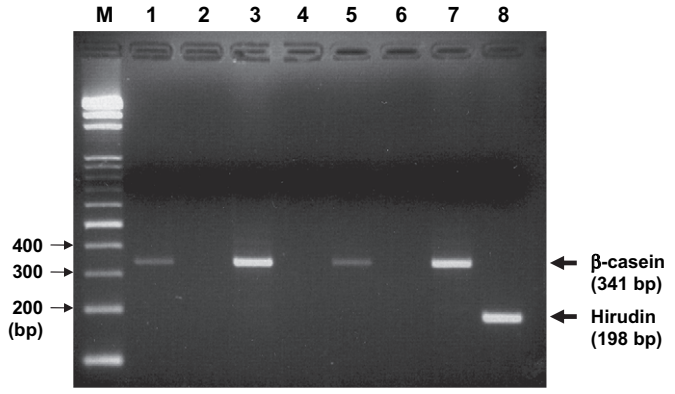


Fig. 4. Hirudin and β-casein gene expression in pGB562/Hi/SI-PMEC cells. The SI-PMEC cells were cultured in basal medium without supplemental prolactin on a petri dish (lanes 1 and 2) and in basal medium with supplemental prolactin on a Matrigel-coated petri dish (lanes 3 and 4). The pGB562/Hi/SI-PMEC cells were cultured in basal medium without supplemental prolactin on a petri dish (lanes 5 and 6) and in basal medium with supplemental prolactin on a Matrigel-coated petri dish (lanes 7 and 8). The amplified β-casein (341 bp) and hirudin (198 bp) DNA fragments are indicated. M, a 100-bp ladder and the molecular masses are indicated.

Of interest in this study was that pGB562/Hi/SI-PMEC cells, which transfected with a mammary gland-specific expression vector pGB562/Hi (Fig. 1A), produced recombinant protein with a molecular mass of 18–20 kDa as determined by Western blotting. In fact, a similar phenomenon was also shown for the commercial recombinant hirudin (Rade et al., 1999). The immunoreactive bands present in the commercial hirudin and the homogenate of SI-PMEC cells did not agree with the predicted 6–7 kDa form of hirudin. It can be proposed that the band was indeed present, with a molecular mass corresponding to that of hirudin trimers identified by the monoclonal anti-hirudin antibody. This speculation is consistent with the report by Rade et al. (1999).

Optimal expression of recombinant hirudin by SI-PMEC cells requires not only stromal Matrigel, which supports differentiation by organizing the basement membrane and providing growth factors, but also stimulation by lactogenic hormones. Our results indicate that the most important factor for enhancing production of recombinant protein in SI-PMEC is Matrigel rather than prolactin. Wu et al. (2003) reported that prolactin plays a major role in inducing milk protein gene expression in the mouse mammary epithelial cell line NMuMG. In our previous study, we showed that prolactin is essential and can strongly increase milk gene expression in primary porcine mammary epithelial cells (Sun et al., 2005). Contrary to the previous results, in the present study we demonstrated that the established porcine mammary epithelial cell line, SI-PMEC, can be triggered to differentiate, form functional structures and express milk genes without prolactin supplementation. However, providing a suitable substratum is necessary for triggering differentiation of SI-PMEC cells. The genetic modifications in the SI-PMEC need to be further explored before the differential regulation of the cell is conclusively understood.

In this study, we focused on producing bioactive recombinant hirudin in SI-PMEC cells. Additional studies are needed to determine optimal conditions for secretion of the recombinant proteins in the SI-PMEC system. The yields of recombinant proteins produced by the immortalized bovine mammary epithelial MAC-T cells are variable. The yield of recombinant equine prorelaxin in MAC-T cells was only 4 ng/ml (Neumann et al., 2006). However, the recombinant spider silk produced by MAC-T cells could reach 20–50 ng/ml (Lazaris et al., 2002). In addition to using the SI-PMEC system to produce recombinant proteins, successful *in vitro* expression of recombinant product in a porcine mammary epithelial cell line also can be used for an *in vitro* screening system to identify superior transgenes prior to transfer, thereby improving the efficiency of transgenic livestock generation to produce pharmaceutically relevant peptides in milk. By targeting expression to

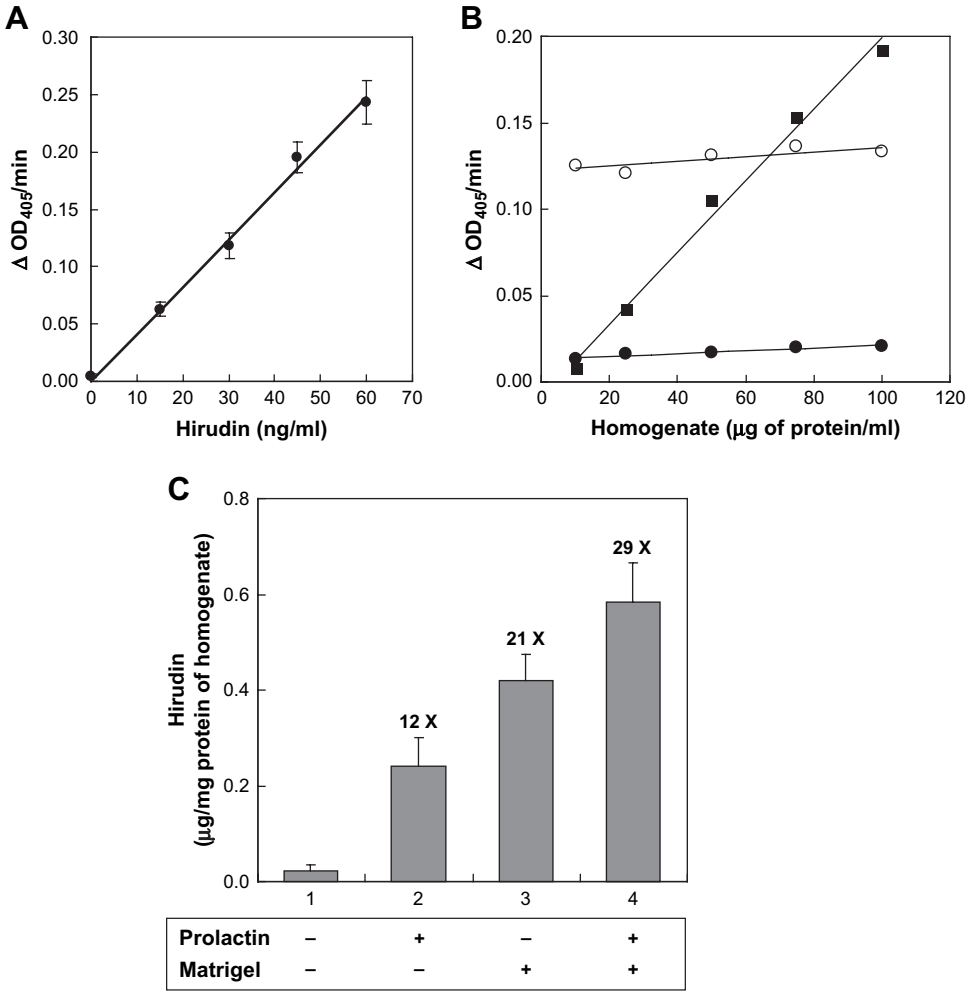


Fig. 5. Anti-coagulation activity assay of recombinant hirudin in the homogenate of pGB562/Hi/SI-PMEC cells and the effects of Matrigel and prolactin on the production of recombinant hirudin in the cells. (A) Thrombin titration curve of average residual thrombin activity plotted against increasing concentrations of the commercial recombinant hirudin from 0 to 60 ng/ml at 15 ng/ml intervals. The equation of the linear relationship between ΔOD_{405} (y) and hirudin mass (x) is $y = 0.0041x$, $R^2 = 0.9955$. (B) The plots of anti-coagulation activity of the homogenates isolated from the undifferentiated pGB562/Hi/SI-PMEC cells (closed circles), the homogenates isolated from the undifferentiated pGB562/Hi/SI-PMEC cells which were artificially added with 30 ng commercial recombinant hirudin to each concentration of the detected samples (open circles), and the homogenates isolated from the differentiated pGB562/Hi/SI-PMEC cells that were cultured in basal medium with supplemental prolactin (5 μ/ml) and grown on a Matrigel-coated dish (closed squares). (C) The anti-coagulation activity of the homogenates isolated from each treatment was determined and the hirudin content was calculated. Treatment 1, the homogenate isolated from pGB562/Hi/SI-PMEC cells cultured in basal medium and grown on a plastic dish; treatment 2, from pGB562/Hi/SI-PMEC cells cultured in basal medium with supplemental prolactin (5 μg/ml) and grown on a plastic dish; treatment 3, from pGB562/Hi/SI-PMEC cells cultured in basal medium and grown on a Matrigel-coated dish; treatment 4, from pGB562/Hi/SI-PMEC cells cultured in basal medium with supplemental prolactin and grown on a Matrigel-coated dish. Each value indicates the mean ± SE of three independent determinations.

the mammary gland, several heterologous recombinant human proteins have been produced from the milk of transgenic livestock, including that of goats, sheep, cattle and rabbits (Houdebine, 2000; Niemann and Kues, 2003). The biological activity of the recombinant proteins such as human anti-thrombin III, α-antitrypsin and tissue plasminogen activator were assessed and the therapeutic effects have been characterized (Meade et al., 1999; Rudolph, 1999). The proteins from the milk of transgenic livestock are expected to be on the market within the next few years (Niemann and Kues, 2003).

In summary, our study reports the potential of gene transfection by electroporation in our established porcine mammary epithelial cell line, SI-PMEC. The hirudin transgene was successfully expressed in differentiated SI-PMEC cells, which formed functional structures. Anti-coagulation bioactivity was detected for the produced recombinant hirudin. Thus, we have provided experimental data to demonstrate that SI-PMEC cells have potential to produce pharmaceutically relevant peptides.

Acknowledgements

The authors gratefully acknowledge Prof. MC Huang at the National Chung Hsing University and Dr ST Wu at the National Chiayi University in Taiwan for the gift of pGB562/GFP plasmid. This work was supported by the grant of 92-EC-17-A-17-R7-0454 from the Ministry of Economic Affairs of Executive Yuan and the grant of NSC 96-2628-B-009-001-MY3 from National Science Council, Taiwan.

References

Dodt J, Müller HP, Seemüller U, Chang JY. The complete amino acid sequence of hirudin, a thrombin specific inhibitor: application of colour carboxymethylation. FEBS Lett 1984;165:180–4.

Ebert KM, DiTullio P, Barry CA, Schindler JE, Ayres SL, Smith TE, et al. Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. Biotechnology (NY) 1994;12:699–702.

German T, Barash I. Characterization of an epithelial cell line from bovine mammary gland. In Vitro Cell Dev Biol Anim 2002;38:282–92.

Gorodetsky SI, Bremel R. The factors of tissue-specific expression of the bovine β-casein gene. In: Castro FO, Janne J, editors. Mammary gland transgenesis: therapeutic protein production. New York: Springer-Verlag; 1998.

Greinacher A. Lepirudin: a bivalent direct thrombin inhibitor for anticoagulation therapy. Expert Rev Cardiovasc Ther 2004;2:339–57.

Groner B, Gouilleux F. Prolactin-mediated gene activation in mammary epithelial cells. Curr Opin Genet Dev 1995;5:587–94. doi:10.1016/0959-437X(95)80027-1.

Guarna MM, Cote HC, Kwan EM, Rintoul GL, Meyhack B, Heim J, et al. Factor X fusion proteins: improved production and use in the release *in vitro* of biologically active hirudin from an inactive alpha-factor-hirudin fusion protein. Protein Expr Purif 2000;20:133–41.

Harvey RP, Degryse E, Stefani L, Schamber F, Cazenave JP, Courtney M, et al. Cloning and expression of a cDNA coding for the anticoagulant hirudin from the bloodsucking leech, Hirudo medicinalis. Proc Natl Acad Sci U S A 1986;83:1084–8.

Houdebine LM. Transgenic animal bioreactors. Transgenic Res 2000;9:305–20.

Kang YK, Lee CS, Chung AS, Lee KK. Prolactin-inducible enhancer activity of the first intron of the bovine β-casein gene. Mol Cell 1998;8:259–65.

Kim MD, Yoo YJ, Seo JH. Enhanced transformation efficiency of an anticoagulant hirudin gene into *Saccharomyces cerevisiae* by a double δ-sequence. J Microbiol Biotechnol 2001;11:61–4.

Kochanowski R, Kotlowski R, Szweda P. Novel method of expression and purification of hirudin based on pBAD TOPO, pTYB12 vectors and gene synthesis. Protein Expr Purif 2006;50:25–30. doi:10.1016/j.pep.2006.06.004.

Lazaris A, Arcidiacono S, Huang Y, Zhou JF, Duguay F, Chretien N, et al. Spider silk fibers spun from soluble recombinant silk produced in mammalian cells. Science 2002;295:472–6.

Lin CS, Hsu CW. Differentially transcribed genes in skeletal muscle of duroc and taoyuan pigs. J Anim Sci 2005;83:2075–86.

Markwardt F. Hirudin as an inhibitor of thrombin. Methods Enzymol 1970;19:924–32.

Matheson AJ, Goa KL. Desirudin: a review of its use in the management of thrombotic disorders. Drugs 2000;60:679–700.

Meade HM, Echelard Y, Ziomek CA, Young MW, Harvey M, Cole ES, et al. Expression of recombinant proteins in the milk of transgenic animals. In: Fernandez JM, Hoeffler JP, editors. Gene expression systems. San Diego, CA: Academic Press; 1999. p. 399–427.

Neumann JL, Lazaris A, Huang YJ, Karatzas C, Ryan PL, Bagnell CA. Production and characterization of recombinant equine prorelaxin. Domest Anim Endocrinol 2006;31:173–85. doi:10.1016/j.domaniend.2005.10.001.

Niemann H, Kues WA. Application of transgenesis in livestock for agriculture and biomedicine. Anim Reprod Sci 2003;79:291–317. doi:10.1016/S0378-4320(03)00169-6.

Pan CH, Lin JL, Lai LP, Chen CL, Huang SKS, Lin CS. Downregulation of angiotensin converting enzyme II is associated with pacing-induced sustained atrial fibrillation. FEBS Lett 2007;581:526–34.

Petitclerc D, Attal J, Théron MC, Bearzotti M, Bolifraud P, Kann G, et al. The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice. J Biotechnol 1995;40:169–78. doi:10.1016/0168-1656(95)00047-T.

Rade JJ, Cheung M, Miyamoto S, Dichek DA. Retroviral vector-mediated expression of hirudin by human vascular endothelial cells: implications for the design of retroviral vectors expressing biologically active proteins. Gene Ther 1999;6:385–92.

Riesbeck K, Chen D, Kemball-Cook G, McVey JH, George AJ, Tuddenham EG, et al. Expression of hirudin fusion proteins in mammalian cells: a strategy for prevention of intravascular thrombosis. Circulation 1998;98:2744–52.

Rijnkels M, Pieper FR. Casein gene-based mammary gland-specific transgene expression. In: Castro FO, Janne J, editors. Mammary gland transgenesis: therapeutic protein production. New York: Springer-Verlag; 1989. p. 41–64.

Roberts B, DiTullio P, Vitale J, Hehir K, Gordon K. Cloning of the goat beta-casein-encoding gene and expression in transgenic mice. Gene 1992;121:255–62.

Rose MT, Aso H, Yonekura S, Komatsu T, Hagino A, Ozutsumi K, et al. *In vitro* differentiation of a cloned bovine mammary epithelial cell. J Dairy Res 2002;69:345–55.

Rosen JM, Wyszomierski SL, Hadsell D. Regulation of milk protein gene expression. Annu Rev Nutr 1999;19:407–36.

Rudolph NS. Biopharmaceutical production in transgenic livestock. Trends Biotechnol 1999;17:367–74. doi:10.1016/S0167-7799(99)01341-4.

Seemüller U, Dodt J, Fink E, Fritz H. Proteinase inhibitors of the leech *Hirudo medicinalis* (hirudins, bdellins, eglins). In: Barrett AJ, Salvesen G, editors. Proteases. Amsterdam: Elsevier; 1986. p. 337–59.

Shi B, Li J, Yu A, Yuan B, Wu B. Two-step ion-exchange chromatographic purification of recombinant hirudin-II and its C-terminal-truncated derivatives expressed in *Pichia pastoris*. Process Biochem 2006;41:2446–51. doi:10.1016/j.procbio.2006.07.018.

Sohn JH, Lee SK, Choi ES, Rhee SK. Gene expression and secretion of the anticoagulant hirudin in *Saccharomyces cerevisiae*. J Microbiol Biotechnol 1991;1:266–73.

Sun YL, Lin CS, Chou YC. Establishment and characterization of a spontaneously immortalized porcine mammary epithelial cell line. Cell Biol Int 2006;30:970–6. doi:10.1016/j.cellbi.2006.06.023.

Sun YL, Lin CS, Chou YC. Gene transfection and expression in a primary culture of mammary epithelial cells isolated from lactating sows. Cell Biol Int 2005;29:576–82. doi:10.1016/j.cellbi.2005.03.021.

Tan S, Wu W, Liu J, Kong Y, Pu Y, Yuan R. Efficient expression and secretion of recombinant hirudin III in *E. coli* using the L-asparaginase II signal sequence. Protein Expr Purif 2002;25:430–6.

Weitz JI, Bates ER. Direct thrombin inhibitors in cardiac disease. Cardiovasc Toxicol 2003;3:13–25.

Wu HT, Lin CS, Huang MC. *In vitro* and *ex vivo* green fluorescent protein expression in alveolar mammary epithelial cells and mammary glands driven by the distal 5′-regulative sequence and intron 1 of the goat beta-casein gene. Reprod Fertil Dev 2003;15:231–9.

Zhou XS, Zhang YX. Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by controlling the specific growth rate. Biotechnol Lett 2002;24:1449–53.