ORIGINAL RESEARCH

The high-level expression analysis of rhPA in the rhPA/gGH double-transgenic rabbits and its thrombolysis activity in vitro

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Abstract: 【Objective】In this study, the rhPA/gGH double transgenic rabbits were constructed, and the expression level of rhPA,rabbit growth and development features were analyzed, which might provide a new idea for obtain rhPA high level expression transgenic animals.【Method】Two rhPA transgenic rabbits fertilized eggs were microinjected with linearized GH plasmid to obtain the rhPA/gGH rabbits.The integration of rhPA/gGH gene was detected by PCR. The rhPA expression level in transgenic rabbit milk was detected by ELISA and Western blotting,and FAPA was performed to detect the in vitro thrombolytic activity of rhPA.The body weight of transgenic rabbits

at different growth stages were measured to test the effect of gGH gene on rhPA/gGH double transgenic rabbits growth and development .【Result】A total of 151 rhPA transgenic rabbits fertilized eggs were obtained through superovulation, 125 of them were microinjected with linearized GH plasmid and transplanted into 8 surrogate mother rabbits.Six surrogate mother rabbits were pregnant, with a pregnancy rate of 75.0% (6/8)， 16 rhPA/gGH gene double transgenic rabbits were identified by PCR (10♂,6♀). The rhPA expression levels in rhPA single-transgenic rabbit whey were 0.27–0.63g/L, while the rhPA expression leves were 4.98-12.24 g/L in the rhPA/gGH double-transgenic rabbits whey. The rhPA expression levels of rhPA/gGH double-transgenic rabbit whey were significantly increased by about 17.2–23.8 times, and had higher thrombolytic activity in vitro. There was no significant difference in body weight between rhPA/gGH double transgenic rabbits, rhPA single transgenic or non-transgenic rabbits from birthday to 10 months age(P>0.05). 【Conclusion】 The rhPA/gGH double transgenic rabbits were successfully constrected, which was proved that the introduction of gGH gene could significantly increase the rhPA expression level in the milk of transgenic rabbits and without affecting the growth and development of transgenic rabbits, which laid a foundation for the preparation of transgenic rabbits with higher recombinant protein expression level in the future, and also provide new ideas and new methods for the establishment of mammary gland bioreactor.

Keywords:rhPA/gCH ;double transgenic rabbit; *rhPA* expression; recombinant protein expression

Introduction

Thrombosis diease is a common and frequent disease that might seriously threaten human health and life, and thrombolytic therapy is one of the most widely used and effective treatment methods in clinical practice[1-3]. The human tissue-type plasminogen activator(tPA) is a serine protease synthesized and secreted by vascular endothelial cells, which can efficiently and specifically dissolve thrombus and it is a good second-generation thrombolytic drug[4-5]. The recombinant human plasminogen activator (rhPA) in this study is a recombinant mutants of natural tPA[6], which belongs to the third generation thrombolytic drugs and has more superior thrombolytic efficacy than natural tPA. Therefore, the study of how to stably improve the rhPA expression level is an important guideline for the development of new thrombolytic drugs.

Currently, exogenous gene expression silencing is an important bottleneck in animal mammary gland bioreactor research. Although some methods such as the use of friendly sites (Rosa26, Hipp11, Pifs501), site-specific targeted integration (ZFNs, TALENs, CRISPR/Cas9), functional gene recombination modification and optimization of cis-acting elements (promoters, introns, enhancers) can overcome or alleviate gene expression silencing, there are still many limitations [11-12]. Therefore,

we need to study new ideal techniques to optimize and improve the exogenous genes expression level of transgenic animal. At present, the strategies to improve the expression level of tPA and rhPA gene are mainly in the modification of the gene itself and optimization of cis-acting elements. For example, Ebert KM et al constructed transgenic goat to expression tPA in mammary gland with active function at 3 μg/mL by modifying the tPA mutant recombinant[13]. Lu Y et al used sheep β-lactoglobulin gene as a promoter regulatory sequence to construct the vector,by which the tPA expressed in the mammary gland of transgenic mice was about 6 μg/mL[14]. Zhou Y et al used tPA to replace the partial coding sequence of mouse whey acid protein to construct the mWAP-htPA hybrid gene base, and the tPA expression level in the transgenic mice mammary gland was increased[15]. In our laboratory, the goat β-casein and CMV were used as the hybrid promoter regulatory sequences to construct a recombinant mammary gland-specific expression vector (PCL25/rhPA). The expression level of recombinant tPA in the mammary gland of rhPA transgenic rabbits could reach 630μg/mL[16-17] . However, the expression level of tPA and rhPA in the above studies is still at a low level and has never been scientifically and effectively solved.

Some researchers have proved that transgenic animals constructed through double gene co-integration can produce synergistic effect, where one gene can promote the expression level of the other gene and increase the expression level of the target gene[18-20], resulting in higher yields. For example, Sendtner M et al[19] found that transfection the double gene with ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) was able to significantly increase the expression level of LIF protein and produce trophic physiological effects on motor neurons. Chen XY et al [20] constructed double transgenic pigs by injected recombinant lentivirus containing fluorescent protein genes (DsRedl and Venus) into 2-cell stage embryos of pigs, and the double transgenic pigs can synergistically promote the efficient fluorescent protein gene expression. Kundu S [21] and Gomes[22] have also concluded that double transgenic co-integration synergistically can promote the expression of the target recombinant protein. However, there are few reports on the use of double transgenic synergy to promote the expression level of tPA gene.

Goat growth hormone (gGH) is a prolactin-like protein secreted by the anterior pituitary gland of goats, which controls the activation of β-casein and α-LA receptors to produce synergistic effects

and has the function of promoting mammary gland growth, development and maintaining lactation [23-25]. This indicates that the transferring of gGH gene into animals may enhance the recombinant protein expression level. Therefore, it may play an important role in transgenic animals. However, there was not been reported whether the double gene co-integration of gGH and rhPA genes into transgenic rabbits can improve the expression of rhPA or not, the gGH gene whether can efficiently synergistically promote the rhPA expression level in the mammary gland of transgenic rabbits deserves further investigation.

In this study, the previously obtained rhPA single transgenic rabbits[16] (PCL25/rhPA mammary gland specific expression vector with rhPA gene) were used as the experimental rabbit. The rhPA transgenic rabbits fertilized eggs were microinjected with gGH gene to construct rhPA/gCH transgenic rabbits, which might provide a new idea and method for the preparation of high expression level rhPA transgenic animals in the future, and also might lay a foundation for the efficient and large-scale production of other recombinant pharmaceutical proteins.

1 Materials and methods

1.1 Vectors and Reagents

PCL25/rhPA (deletion mutants of L, F, E and K1 regions of tPA) and PCL25/gGH plasmid and strains were preserved in the laboratory. They were mammalian mammary gland-specific expression vectors with goat β-casein as regulatory element and CMV as promoter (Fig.1), which have been validated for expression recombinant protein on goat cells, mice, and individual rabbits[16,26-27]. FSH (Ningbo Sansheng Pharmaceutical Co. Ltd.), HCG (Lizhu Pharmaceutical Co. Ltd.), Sumianxin II(Veterinary Institute of Military University), FBS(HyClone), hyaluronidase(Sigma), Zoletil50(Virbac), protease K(Sigma), M2(Sigma), M16 (Sigma), mouse anti-tPA monoclonal antibody (Santa Cruz), goat anti-mouse monoclonal antibody IgG-HRP(Santa Cruz); DNA gel purification and recovery kit was purchased from QIAGEN, various restriction enzymes and DNA polymerases were purchased from Takara Bio (Dalian) Co. Ltd., other reagents were purchased from Shanghai Pharmaceuticals, Shanghai Shenggong Bioengineering Co. Ltd., Nanjing Shengxing

Biotechnology Co. Ltd.

1.2 Animals

The rhPA single transgenic rabbits (New Zealand rabbits, labeled K29 and K34, with goat β-casein gene as a regulatory element) [16] and normal non-transgenic New Zealand rabbits were raised with single cages in Jiangsu Province Transgenic Animal Pharmaceutical Engineering Research Center, with a temperature of 20°C, 12h of light (7:00-19:00) and free of food intake . All animal procedures and study designs were conducted under the Guide of the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China) and approved by the Animal Care and Use Committee of Yangzhou University, Yangzhou, China (license number: SYXK(Su)2017-0044).

1.3 Preparation of gene fragments for microinjection

PCL25/gGH plasmids were linearized by Not/Sal Ⅰ endonuclease double digestion, the gene fragments were recovered for microinjection by using QIAGEN DNA gel purification recovery kit. The gene fragments were diluted to 5 $ng \cdot \mu L^{-1}$ using TE buffer (5 $mmol \cdot L^{-1}$Tris, pH 7.4 0.1 $mmol \cdot L^{-1}$ EDTA) and stored at -20°C.

1.4 Construction of rhPA/gGH transgenic rabbits

The rhPA transgenic rabbits (K29, K34) selected as donors and FSH were injected intramuscularly into the hindlimbs muscle with 10 IU/each rabbit in the morning and evening (12 h interval) for 3 d. On the 4th day, 5 IU/each FSH was injected intramuscularly at 7:00 a.m and 100 IU/each hCG was injected intravenously into the ear margins at 19:00 p.m.to obtain fertilized eggs. On the 5th day at 12:00 noon, fertilized eggs were collected by surgery[16].The rabbits were anesthetized with subcutaneous atropine 1 $mg \cdot kg^{-1}$ and intravenous injection of zoletil-50 7.5 $mg \cdot kg^{-1}$ at the ear margin.The gGH gene fragments microinjected fertilized eggs were incubated in an at 38°C, 5% CO_2 saturated humidity incubator for 30 min. Then the fertilized eggs were

transplanted into the oviducts of synchronized estrous recipient female rabbits with 10-30 eggs each to be pregnant. The process of transgenic rabbit surgery was shown in Figure 2.

1.5 PCR detection of transgenic rabbits

The ear tip tissue of newborn rabbits was cut aseptically about 1-2 mm^3, and added tissue lysate containing with 250 μg proteinase K, which digested overnight at 55°C. The genome was extracted by phenol/chloroform extraction and precipitated by 100% ethanol for PCR detection.Two pairs of primers for both rhPA and gGH genes were designed (as shown in Figure 1 and Table 1), in which CtPA-F/R primers were used for rhPA gene detection, and the PCR procedures were: 94℃ pre-denaturation for 5 min; 94℃ denaturation for 1 min, 50℃ annealing for 45s, 72℃ extension for 45s, a total of 30 cycles; 72℃ extension for 5 min. CgGH-F/R primers were used for gGH gene detection, and the PCR parameters were: 94℃ pre-denaturation for 5min; 94℃ denaturation for 1min, 54℃ annealing for 45s, 72℃ extension for 1min, a total of 30 cycles; 72℃ extension for 5min. PCR amplification products were subjected to 1% agarose gel electrophoresis to determine whether the band size was correct.

Table 1 The primer sequences for PCR

Primer name	**Primer sequence (5′–3′)**	Product Size (bp)
CgGH-F	TCGAGCGGATGATGGCTGCAGGCCCCCGG	572
CgGH-R	CGAGCGGCTAGAAGGCACAGCTGGCCTCC	
CtPA-F	GTCGTGGATAGCGGTTTGATGAGACG	655
CtPA-R	CAGAGCCCTCCTTTGATGCTGATCGC	

1.6 ELISA detection of rhPA expression level

Transgenic female rabbits were mated with male rabbits and collected milk. The milk was centrifuged at 10,000×g for 30 min to remove the upper fat layer and the lower turbid layer, then the

whey was diluted 100 times in PBS for detection.100 μL whey and 100 μL coating buffer (1.696 $g \cdot L^{-1}$ Na_2CO_3, 2.856 $g \cdot L^{-1}NaHCO_3$, pH 9.6) were added to each well in 96-well ELISA plate overnight at 4°C. The coating buffer was discarded and washed 3 times with PBS containing 0.05% Tween-20.Add 200 μL of sealing fluid (PBS containing 10% fetal bovine serum)to each well and incubated at 37 ℃ for 2 h. The mouse anti-tPA monoclonal antibody was used as the primary antibody (sc-59721, Santa Cruz), and the goat anti-mouse monoclonal antibody IgG-HRP was used as the secondary antibody (sc-2005, Santa Cruz).All of the antibody was incubated respectively at 37 ℃ for 2 hours. 50 μL of chromogenic reagent (5 mg OPD, 15 μL 30% H_2O_2,28.4 $g \cdot L^{-1}Na_2HPO_4$, 19.2 $g \cdot L^{-1}$ citric acid) was added to each well and incubated in dark at 37°C for 20min. After coloration, the OD_{450} value was measured by microplate reader. Alteplase was used as a standard to draw a standard curve,then calculate rhPA expression level in rhPA/gGH double-transgenic rabbits and rhPA single-transgenic rabbits.

1.7 Western blotting

Transgenic rabbit whey was diluted with 100-fold PBS and subjected to 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) [16]. The acrylamide gel was transferred to PVDF membrane using transfer buffer (1.93 $g \cdot L^{-1}$tris, 9 $g \cdot L^{-1}$glycine) at 250 mA for 3.5 h. After washing with ultrapure water, the gel was blocked with blocking buffer (20 $mmol \cdot L^{-1}$Tris, 137 $mmol \cdot L^{-1}$NaCl,0.1% Tween-20,10% fetal bovine serum, pH7.6) at 37°C for 2h. PVDF membrane was incubated with the primary antibody (1:2000 dilution, mouse anti-tPA monoclonal antibody, sc-59721, Santa Cruz) for 2 h at 37°C. After 3 times wash with TTBS (20 $mmol \cdot L^{-1}$Tris, 137 $mmol \cdot L^{-1}$NaCl, 1% Tween-20, pH7.6),PVDF membrane was incubated with the secondary antibody-HRP dilution (1:2000 dilution, goat anti-mouse monoclonal antibody IgG-HRP, sc-2005, Santa Cruz) at 37℃ for 2h. The PVDF membrane was removed, washed with PBS, added chromogenic reagent (50mg DAB, 100mL 0.05 $mol \cdot L^{-1}$ TB, 30μL 30% H_2O_2, pH7.6), and incubated at room temperature for 15 min, air-dried, photographed, recorded and stored.

1.8 FAPA assay for thrombolytic activity

Using PBS buffer as solvent, 1% agarose gel, 10mg/mL fibrinogen and 10U/mL thrombin were prepared respectively. Boil and melt 1% agarose gel, and take 20mL in a 50mL centrifuge tube, until the temperature dropped to about 50°C without scalding. Warm 1mL of 10mg/mL fibrinogen preheated to 37℃ and add to agarose gel,then take 1mL of 10U/mL thrombin preheated to 42℃ and add to agarose gel. After agarose gel solidification at room temperature, punch and seal the bottom, and added 50 μL of rabbit whey into each well,alteplase as a positive control, normal non-transgenic rabbit whey as a negative control, and PBS as a blank control. The wells were placed at 37℃ overnight to measure the sizes of the transparent rings.

1.9 Growth and development monitoring of rhPA/gGH double transgenic rabbits

Under the same weaning time and feeding management conditions, the body weight of rhPA single-transgenic rabbits, rhPA/gGH double-transgenic rabbits and normal non-transgenic rabbits (same strain, both are New Zealand rabbits) at different growth and development stages were monitored. The body weight of rabbits was measured continuously from birth to 10 months age, and the growth curves of rabbits were plotted with time (month) as the horizontal coordinate and weight (g) as the vertical coordinate, and the significance of differences was analyzed. The growth and development of rhPA single-transgenic rabbits, rhPA/gGH double-transgenic rabbits and normal non-transgenic rabbits were analyzed.

1.10 Statistical analysis of data

The statistical software SPSS 25.0 was applied to process and statistically analyze the data. The experimental datas were expressed as mean ± standard error ($\bar{x}\pm s$), and the one-way ANOVA and t-test were performed, the difference was statistically significant when $P<0.05$ and the difference was not statistically significant when $P\geq0.05$.

2 Results

2.1 Purification and recovery of PCL25/gGH gene fragments for rabbit fertilized eggs microinjection

The plasmid PCL25/gGH was double digested with Not I/Sal I enzyme, and the electrophoresis patterns of the recovered gene fragments using the gel purification recovery kit are shown in Figure 1. A bright band about 16 700 bp can be seen from the figure and no other band can be found. The electrophoresis results showed that the gene fragment about 16 700bp was successfully digested and recovered for microinjection.

2.2 Screening and analysis of double transgenic rabbits

A total of 174 rabbit fertilized eggs were obtained from 2 rhPA single transgenic rabbits (labeled K29 and K34) by supernumerary ovulations, of which 151 were fertilized, with a fertilization rate 90.2% (157/174). The 144 better fertilized eggs were selected for microinjection and transplanted into the oviducts of synchronized estrous recipient female rabbits,six of them became pregnant and delivered successfully, with a pregnancy rate of 75.0% (6/8). A total of 40 rabbits were born. A total of 25 transgenic rabbits integrated with the rhPA gene by PCR detection. 16 rabbits (10♂,6♀) integrated with the rhPA/gGH double gene (Fig.3), indicating these rabbits are rhPA/gGH double-transgenic rabbits. The rhPA/gGH double gene integration rate was 40.0% (16/40),which was detailed in Table 2.

Table 2 Statistics of rhPA expression level in mammary glands of single and double transgenic rabbits

Samples	Single-transgenic		rhPA/gGH Double-transgenic					
	K29	K34	K29-1	K29-2	K34-1	K34-2	K34-3	K34-4
rhPA expression levels (g/L)	0.63	0.27	10.83	12.24	6.15	5.21	6.43	4.98

Expressing relative multiple of rhPA (Double-transgenic/ Single-transgenic)	-	-	17.2 (10.83/0.63)	19.4 (12.24/0.63)	22.8 (6.15/0.27)	19.3 (5.21/0.27)	23.8 (6.43/0.27)	18.4 (4.98/0.27)
Average expression levels (g/L)	0.45±0.25*				7.64±1.05*			

*There was significant difference between single-transgenic, rhPA/gGH double-transgenic and normal non transgenic rabbits in the same column ($P < 0.05$).

2.3 ELISA assay

The results of the rhPA expression levels of transgenic rabbits whey by ELISA are shown in Figure 4 and Table 2. The expression levels of rhPA in the whey of K29 single-transgenic rabbit were 0.63g/L, and the expression levels of rhPA in the K29-1 and K29-2 rhPA/gGH double-transgenic rabbits whey were 10.83 and 12.24 g/L, respectively. The rhPA expression level of double-transgenic rabbits was about 19.4 times more than single-transgenic rabbits (12.24/0.63). The rhPA expression level in K34 single-transgenic rabbit whey was 0.27g/L, and the rhPA expression levels of K34-1~K34-4 rhPA/gGH double-transgenic rabbits whey were 6.15g/L, 5.21g/L, 6.43g/L, 4.98g/L, respectively. The rhPA expression level increased by about 23.8 times (6.43/0.27). The rhPA expression level in two rhPA single-transgenic rabbits was 0.27-0.63g/L, while the rhPA expression level in rhPA/gGH double-transgenic rabbits was 4.98-12.24g/L, which was increased by about 17.2-23.8 times. ELISA results showed that the rhPA expression levels in the rhPA/gGH double-transgenic rabbits was significantly higher than that of rhPA single-transgenic rabbits（$P<0.05$）, and gGH gene could synergistically promote the high expression of rhPA gene in the mammary gland of transgenic rabbits.

2.4 Western blotting assay

The results of Western blotting assay of single and double transgenic rabbit whey are shown in Figure 5. A band of 39.2 kD in size can be seen, which is the same size as the band of the positive control. The results indicated that the 39.2 kD protein successfully expressed in this transgenic rabbit whey was the target product rhPA, and its protein molecular weight was correct in size and

consistent with the target protein gene.

2.5 Thrombolytic activity analysis by FAPA

The thrombolytic activity of rhPA expressed in single and rhPA/gGH double transgenic rabbits whey in vitro were detected by fibrin agarose plate assay (FAPA). The ability of rhPA thrombolytic activity in vitro could be preliminarily judged according to the diameter of transparent circle. From Figure 6, it was found that the rhPA expressed in the positive control standard and all the single and double transgenic rabbits whey had thrombolytic activity in vitro, and there were different degrees of thrombolytic transparent circles. However, the PBS blank control group and the normal non-transgenic rabbit whey did not have thrombolytic function, and there were no thrombolytic transparent circles. The rhPA expressed level in the whey of K29-1 and K29-2 rhPA/gGH double transgenic rabbits were higher than that of K29 single transgenic rabbit.

2.6 Growth and development of rhPA/gGH double transgenic rabbits

The body weights of transgenic rabbits integrated with rhPA single gene and rhPA/gGH double gene were measured continuously from month age up to the 10th month and compared with those of normal non-transgenic rabbits (Table 3). There were no significant difference in body weights at different growth stages, which were not statistically significant ($P > 0.05$). In addition, the monthly body weight of all rabbits increased significantly from 0 to 6 months, and there were significant difference in monthly body weight ($P<0.05$). After 7 months, the rabbits monthly weight difference were not significant ($P>0.05$). The growth curves of rabbits (Figure 7) weight showed that the introduction of gGH gene did not affect the normal growth and development of rabbits. Compared with rhPA single-transgenic rabbits and normal non-transgenic rabbits, the weight growth trend of rhPA/gGH double-transgenic rabbits was consistent. After 7 months, the weight growth tends to be gentle, and the average weight of rabbits grown to 10 months were between 4.5-5.0 kg. It can be found when rabbits grew to adulthood (10 months old), there were no significant difference in body

weight between rhPA/gGH double genes, rhPA single transgenic rabbits and normal non-transgenic rabbits (P>0.05)(Fig.8). The results indicate that the transfer of gGH gene did not affect the normal growth and development of the transgenic rabbits, and the rhPA/gGH double transgenic rabbits were able to survive and grow normally to adulthood.

Table 3 Weight measurement of normal rabbits and transgenic rabbits at different growth stages($\bar{x}\pm s$)

Month（M） Weight(g)	0	1	2	3	4	5	6	7	8	9	10
normal non-transgenic rabbit	54±6[a]	621±22[b]	1512±102[c]	2210±113[d]	3123±310[e]	4086±356[f]	4504±67[g]	4587±147[g]	4672±212[g]	4766±105[g]	4833±91[g]
rhPA single-transgenic rabbit	67±12*[a]	674±51*[b]	1595±66*[c]	2242±201*[d]	3287±132*[e]	4161±104*[f]	4486±283*[g]	4534±136*[g]	4599±84*[g]	4691±267*[g]	4753±162*[g]
rhPA/gGH double-transgenic rabbit	61±9*[a]	593±47*[b]	1486±23*[c]	2283±75*[d]	3190±112*[e]	3998±205*[f]	4541±124*[g]	4612±289*[g]	4665±168*[g]	4726±130*[g]	4790±154*[g]

*There was no significant difference between rhPA/gGH and normal non transgenic rabbits (P > 0.05);

3 Discussion

According to the statistical analysis of the World Health Organization (WHO), the annual number of deaths due to cardiovascular diseases is about 13 million worldwide, among which thrombotic diseases account for more than 50%, and there is an obvious increasing trend [28-29]. At present, thrombolytic drugs such as alteplase (tPA), reteplase, monteplase, lanoteplase, and tenecteplase are mainly used in clinical treatment of thrombosis[30]. The recombinant human fibrinogen activator (rhPA) in this study is a newly developed third-generation recombinant thrombolytic drug with the advantages of high efficiency, safety, specificity, small side effects and so on. The clinical use of thrombolytic drugs is mostly produced by prokaryote or mammalian cell expression, which has the limitations of low production or high price, and the popularization of mass use has been limited [16,31]. Therefore, how to efficiently and conveniently produce rhPA at low cost and higher activity has always been a hot topic in scientific research. Since the successful expression of human α-antitrypsin in sheep mammary gland by Wright et al in the 1990s[32], mammary gland

304 bioreactors have shown an attractive prospect, which provides a great possibility for the production
305 of recombinant thrombolytic drugs. However, rhPA and tPA is a non milk protein, and its expression
306 levels in animal mammary glands is low [13-17]. Therefore, it is particularly important to explore how
307 to improve the expression level of non-lactoprotein rhPA in animal mammary glands.

308 At present, there are many methods to improve the efficiency of exogenous gene expression in
309 animals [11-12,33]. The two genes in double transgenic organisms can produce synergistic promotion to
310 regulate the gene network system of the organism, and to increase the expression level of exogenous
311 target genes [34]. In recent years, there have been many reports on double genes to improve the
312 expression level of exogenous target genes. For example, Kundu S et al[21] introduced
313 NUP98-PHF23 (NP23) and NUP98-HOXD13 (NHD13) gene expression vectors into mice to
314 prepare NP23-NHD13 double transgenic mice, and successfully expressed high-level target genes.
315 The studies of Chen XY et al [20] and Sendtner M et al [19] on pig and human somatic cells also
316 proved the phenomenon of double gene pro-expression, which led to a significant increase in the
317 expression level of target genes. Therefore, double gene synergistic pro-expression is a good strategy
318 to increase target gene expression, which provides a new idea for improving the expression of rhPA
319 level in transgenic rabbit mammary glands.

320 Since 1920, when EVANS first demonstrated that the growth-promoting substance in the
321 pituitary gland is growth hormone, scholars have studied the GH gene extensively and intensively,
322 and have achieved important results [35]. It has been reported that growth hormone (GH) is able to
323 combine with the HRE sequence of the β-casein gene to promote receptor activation and
324 synergistically increase the specific expression of lactoproteins [25,36]. Therefore, it is highly possible
325 to improve the expression level of exogenous genes in transgenic animal mammary gland by using
326 GH gene introduction. However, the current strategy to improve the expression level of tPA and
327 rhPA genes in the mammary gland of transgenic animals is often to optimize the gene vector
328 construction [15-16,26-27]. There are few reports on the synergistic promotion of tPA gene expression by
329 double transgenic animals, especially the study of GH gene synergistically promoting the expression
330 of tPA in transgenic animals at home and abroad.

Rabbit is one of the most widely used experimental animals in life science. It is also a model organism commonly used in transgenic experiments. Compared with large animals such as cattle or goat, it has the advantages of more ovulation, short pregnancy, strong fecundity and more estrus throughout the year. Compared with mice, it has the advantages of high lactation and suitable for the production of recombinant medical proteins, which can fill the “blank” between large and small animals [16,37]. The rhPA single transgenic rabbits (goat β-casein gene as the regulatory sequence and verified expression) were selected as the donor rabbits [16] , and 151 fertilized eggs were obtained by superovulation via FSH/hCG. The gGH gene was injected into the pronucleus of fertilized egg by microinjection, and then transplanted into the synchronous estrus female New Zealand recipient rabbits, respectively. Forty rabbits were successfully delivered. Sixteen rhPA/gGH double transgenic rabbits (10♂,6♀) were obtained by PCR integration detection, and the double gene integration rate reached 40.0%, which was consistent with the integration efficiency of transgenic rabbits reported at home and abroad [16,38-41]. The mammary gland expression levels of the six rhPA/gGH double transgenic female rabbits showed that rhPA expressed level in whey were about 4.98-12.24g/L, which were much higher than those of rhPA single transgenic rabbits (0.27-0.63 g/L), and increased by about 17.2-23.8 times. Moreover, the strong thrombolytic activity of rhPA expressed in their whey was demonstrated by thrombolytic activity assay in vitro. The result demonstrated that the introduction of gGH gene can greatly promote the expression level of rhPA gene in the mammary gland of transgenic rabbits, and the expression level and thrombolytic activity in our study was significantly better [13-17,26-27].

In addition, many studies on GH transgenic animals have focused on the ability of growth hormone to regulate the growth of the organism, resulting in a "super" species with an individual size exceeding that of the general wild type [42]. However, the rhPA/gGH double transgenic rabbits obtained in this study showed that the gGH gene did not affect the growth and development of transgenic rabbits by comparing their growth and development with normal non-transgenic rabbits, and the transgenic rabbits with integrated gGH were able to grow and develop normally into adulthood. In general, the body weight of New Zealand adult rabbits is 4.0-5.0 kg [43]. In our study, the six double transgenic rabbits were continuously monitored for 10 months, and it was found that

there was no significant difference in body weight between the transgenic rabbits,rhPA transgenic and normal non-transgenic rabbits at different stages of growth and development. The body weights were 4.5–5.0 kg at the age of 7–10 months, and the weight growth was not obvious. It was speculated that the transgenic rabbits had the same growth pattern as the normal rabbits. The analysis of the results proved that the gGH introduced in this experiment did not affect the growth and development of the transgenic rabbits. The reason may be due to the fact that the gGH selected for the experiment derived from goats rather than rabbits, which could not produce physiological effects similar to those in goats and did not affect the growth and development of the rabbits. Moreover, the gene expression is a multifaceted effect involving integration sites, epigenetics, copy number of exogenous gene, relevant hormone levels and gene networks [44-46]. Therefore, the related studies still need to be continued.

4 Conclusion

The successful preparation of rhPA/gGH double transgenic rabbits by secondary transgenic not only ensured the integration rate of the double genes, but also made the rhPA expression level more comparable. Through the monitoring of the expressed rhPA content in rabbits whey and the body weight at different growth and development stages, it was proved that the rhPA/gGH double transgenic rabbits could significantly increase the expression of rhPA in the mammary gland and maintain a high level of thrombolytic activity. At the same time, the introduced gGH had no significant effect on the growth and development of rabbits, which laid a foundation for the preparation of high-expression transgenic rabbits and other animals in the future, and also provided a new technology and method for the establishment of transgenic animal mammary gland bioreactors and transgenic breeding.

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Disclosure Statement

Shaozheng Song and Yaoling Luo are the first author,Zhengyi He and Junsong Ye, Zhengyi He is the first correspondence author.and all the authors report no declarations of interest conflict. All authors reviewed and approved to be accountable for all aspects of the final manuscript.

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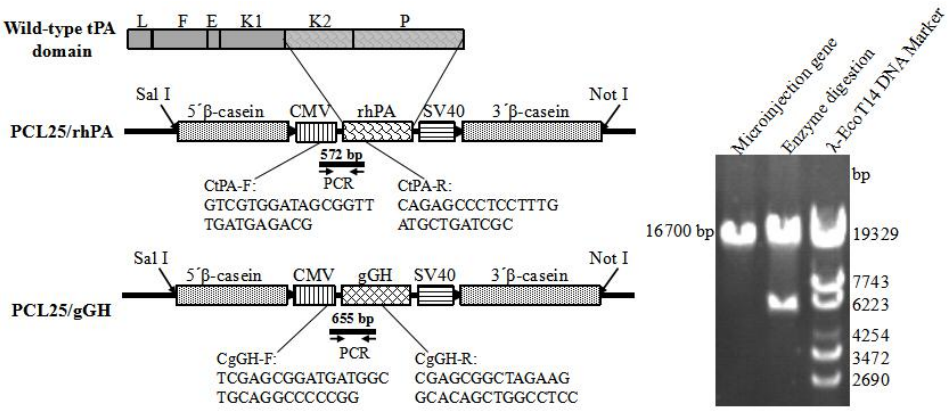

Fig. 1 The structural diagram of PCL25/rhPA and PCL25/gGH mammary gland specific expression vector and enzyme digestion map of PCL25/gGH

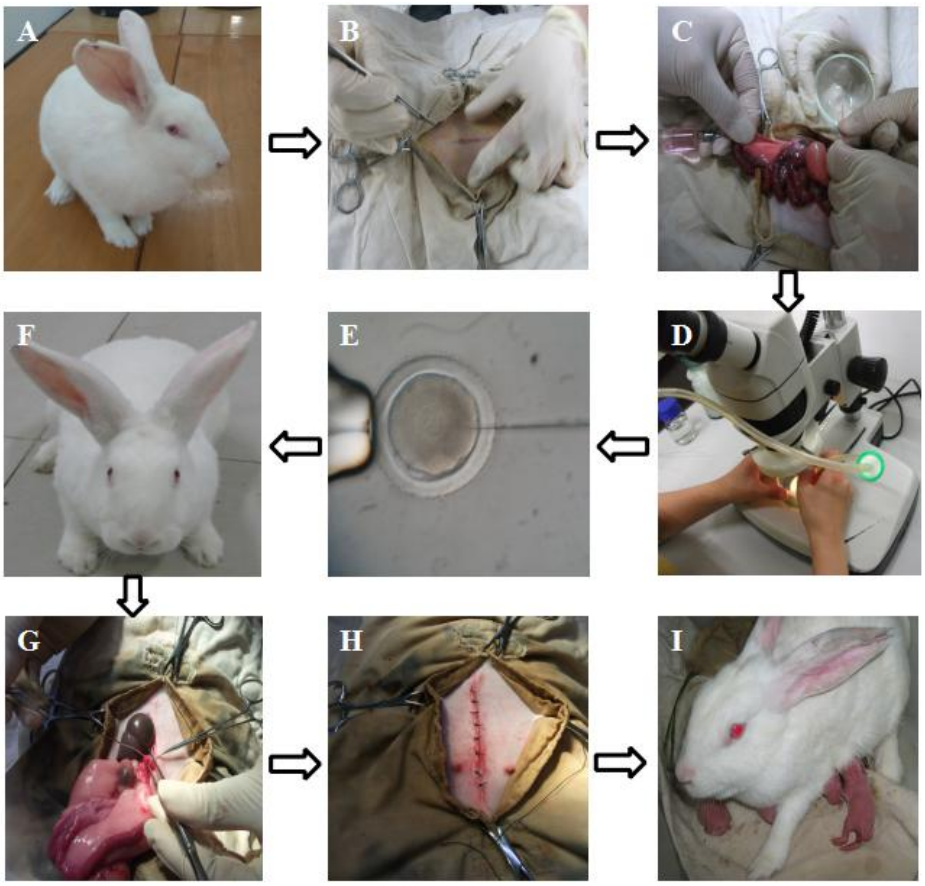

Fig. 2 The preparation flow-process diagram of transgenic rabbit

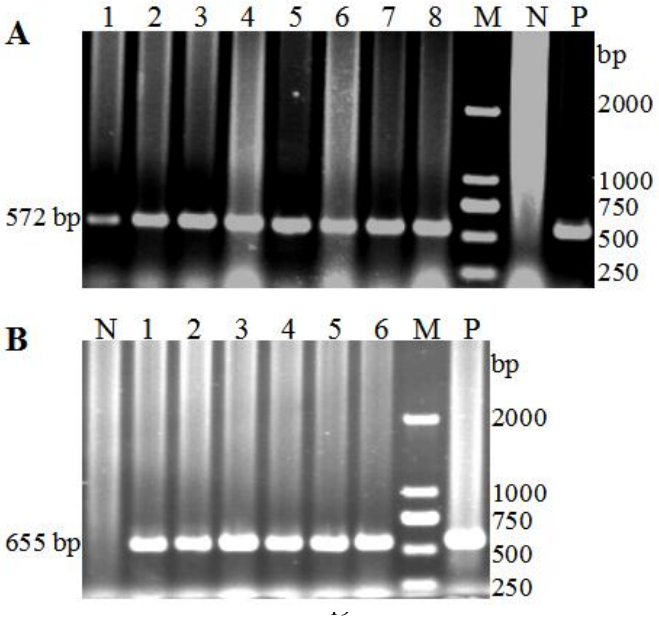

Fig. 3 Electrophoresis of transgenic rabbits by PCR integration detection

A: PCR integration detection of rhPA gene. 1-8: rhPA transgenic rabbits; P: PCL25/rhPA plasmid (positive control); N: Normal non-transgenic rabbits (negative control); M:DL2000 Marker.

B: PCR integration detection of gGH gene. 1-6: rhPA/gGH double transgenic rabbits; P: PCL25/gGH plasmid (positive control); N: Normal non-transgenic rabbits (negative control); M:DL2000 Marker.

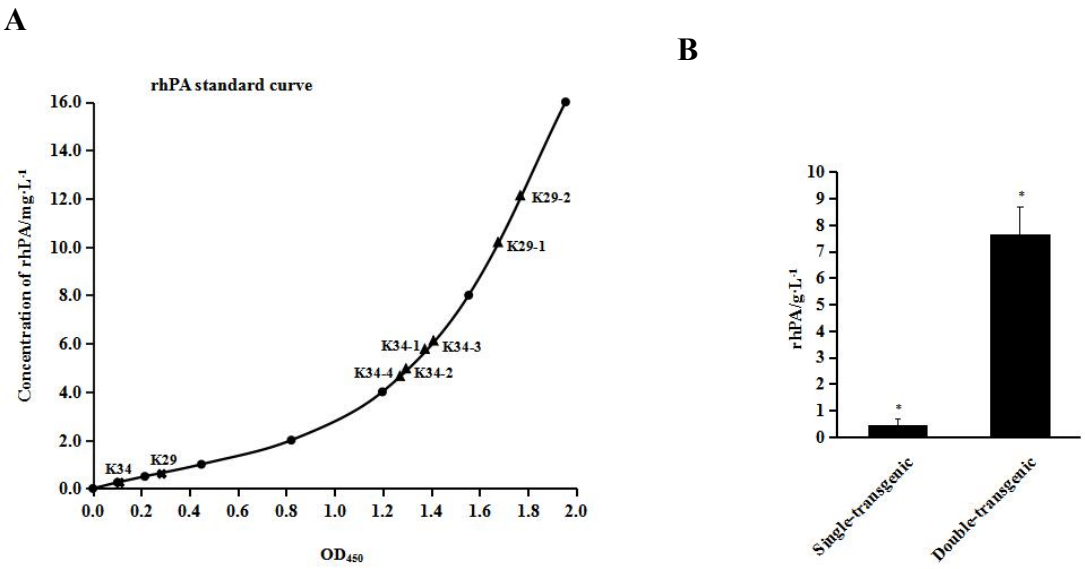

Fig. 4 The expression and analysis of rhPA in mammary glands of transgenic rabbits

A: The standard curve of rhPA expression level in mammary glands of transgenic rabbits. The OD450 value was used as the abscissa and the concentration of alteplase standard (mg · L-1) as the ordinate. The concentration of ateplase were 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 $mg \cdot L^{-1}$, respectively. All rabbits whey were diluted 1000 times with PBS.

B: The expression level of tPA in mammary gland of transgenic rabbits. * $P < 0.05$ compared with rhPA single transgenic rabbits.

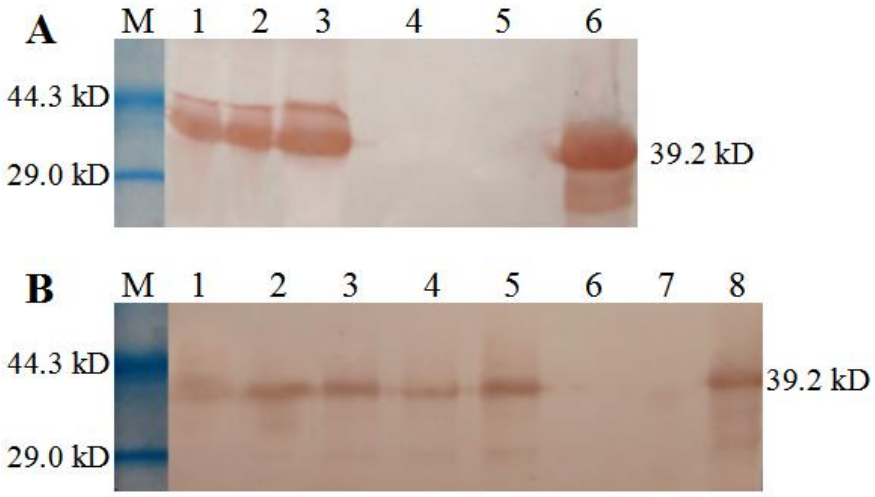

Fig. 5 Western blotting detection results of single and double transgenic rabbits whey

All rabbits whey were diluted 1000 times with PBS.

A: M: Protein molecular weight standard;1:K29 transgenic rabbit; 2:K29-1 transgenic rabbit; 3:K29-2 transgenic rabbit; 4: Normal non-transgenic rabbit; 5:PBS;6: tPA standard.

B: M: Protein molecular weight standard;1:K34 transgenic rabbit; 2:K34-1 transgenic rabbit; 3:K34-2 transgenic rabbit; 4:K34-3 transgenic rabbit; 5:K34-4 transgenic rabbit; 6: Normal non-transgenic rabbit; 7:PBS;8: tPA standard.

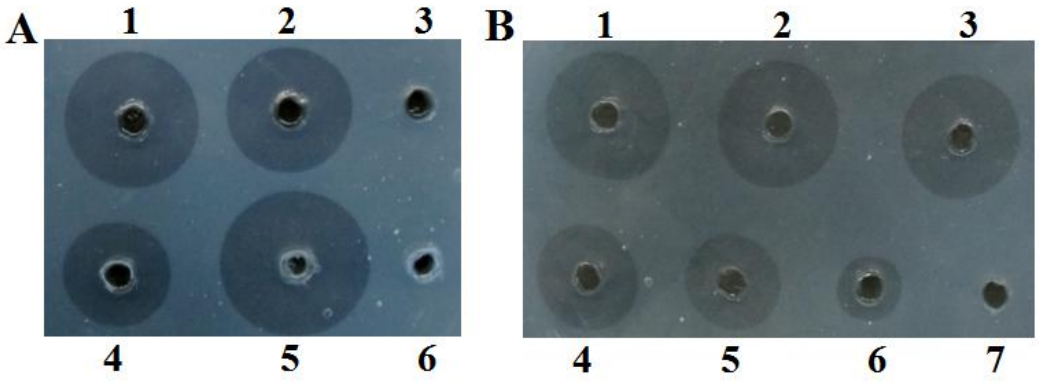

Fig.6

The detection of rhPA activity function in transgenic rabbit whey by FAPA

All whey were diluted 1000 times with PBS.

A:1: K34-1 transgenic rabbit; 2: 1.0 mg/L alteplase standard; 3: PBS; 4:K34 transgenic rabbit; 5:K34-2 transgenic rabbit; 6: Normal non-transgenic rabbit.

B:1: K29-3 transgenic rabbit; 2: K29-1 transgenic rabbit;3:K29-2 transgenic rabbit;4:K29-4 transgenic rabbit;5:1.0 mg/L alteplase standard; 6:K29 transgenic rabbit; 7: Normal non-transgenic rabbit.

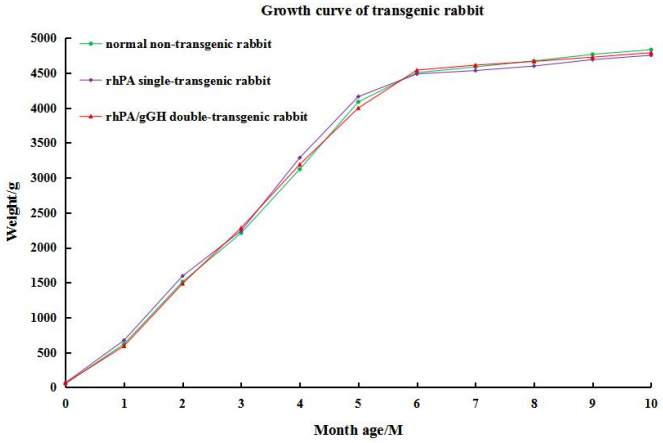

Fig. 7 The growth curve of transgenic rabbits

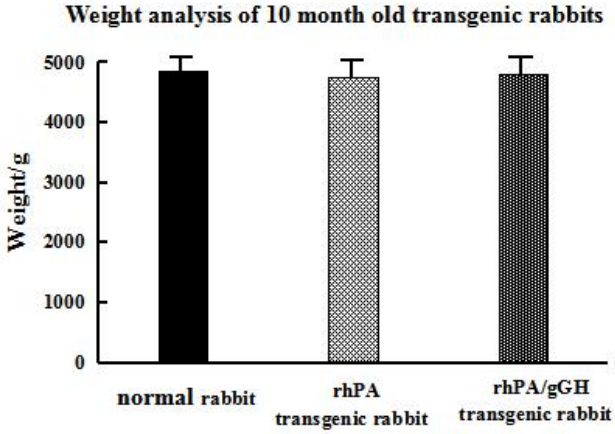

Fig. 8 The comparative analysis of body weight of 10 month old transgenic rabbits（P>0. 05）

Table 1 The primers sequences of PCR

Primer name	**Primer sequence (5′–3′)**	Product Size (bp)
CgGH-F	TCGAGCGGATGATGGCTGCAGGCCCCCGG	572
CgGH-R	CGAGCGGCTAGAAGGCACAGCTGGCCTCC	

CtPA-F	GTCGTGGATAGCGGTTTGATGAGACG	655
CtPA-R	CAGAGCCCTCCTTTGATGCTGATCGC	

613

614 Table 2 Statistics of rhPA expression in mammary glands of single and double transgenic rabbits

Samples	**Single-transgenic**		***rhPA/gGH* Double-transgenic**					
	K29	**K34**	**K29-1**	**K29-2**	**K34-1**	**K34-2**	**K34-3**	**K34-4**
rhPA expression levels (g/L)	0.63	0.27	10.83	12.24	6.15	5.21	6.43	4.98
Expressing relative multiple of rhPA (Double-transgenic/ Single-transgenic)	-	-	17.2 (10.83/0.63)	19.4 (12.24/0.63)	22.8 (6.15/0.27)	19.3 (5.21/0.27)	23.8 (6.43/0.27)	18.4 (4.98/0.27)
Average expression levels (g/L)	0.45±0.25*		7.64±1.05*					

615 *There was significant difference between and normal non transgenic rabbits in the same column (P <0.05).

616

617 Table 3 Weight measurement of normal rabbits and transgenic rabbits at different growth
618 stages($\bar{x}\pm s$)

Month（M） Weight(g)	0	1	2	3	4	5	6	7	8	9	10
normal non-transgenic rabbit	54±6^{a}	621±22^{b}	1512±102^{c}	2210±113^{d}	3123±310^{e}	4086±356^{f}	4504±67^{g}	4587±147^{g}	4672±212^{g}	4766±105^{g}	4833±91^{g}
rhPA single-transgenic rabbit	67±12*^{a}	674±51*^{b}	1595±66*^{c}	2242±201*^{d}	3287±132*^{e}	4161±104*^{f}	4486±283*^{g}	4534±136*^{g}	4599±84*^{g}	4691±267*^{g}	4753±162*^{g}
rhPA/gGH double-transgenic rabbit	61±9*^{a}	593±47*^{b}	1486±23*^{c}	2283±75*^{d}	3190±112*^{e}	3998±205*^{f}	4541±124*^{g}	4612±289*^{g}	4665±168*^{g}	4726±130*^{g}	4790±154*^{g}

619 *There was no significant difference between and normal non transgenic rabbits in the same column (P > 0.05);
620 There was significant difference between superscripts of different English letters in the same line (P < 0.05).

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623