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Transgenic Animals

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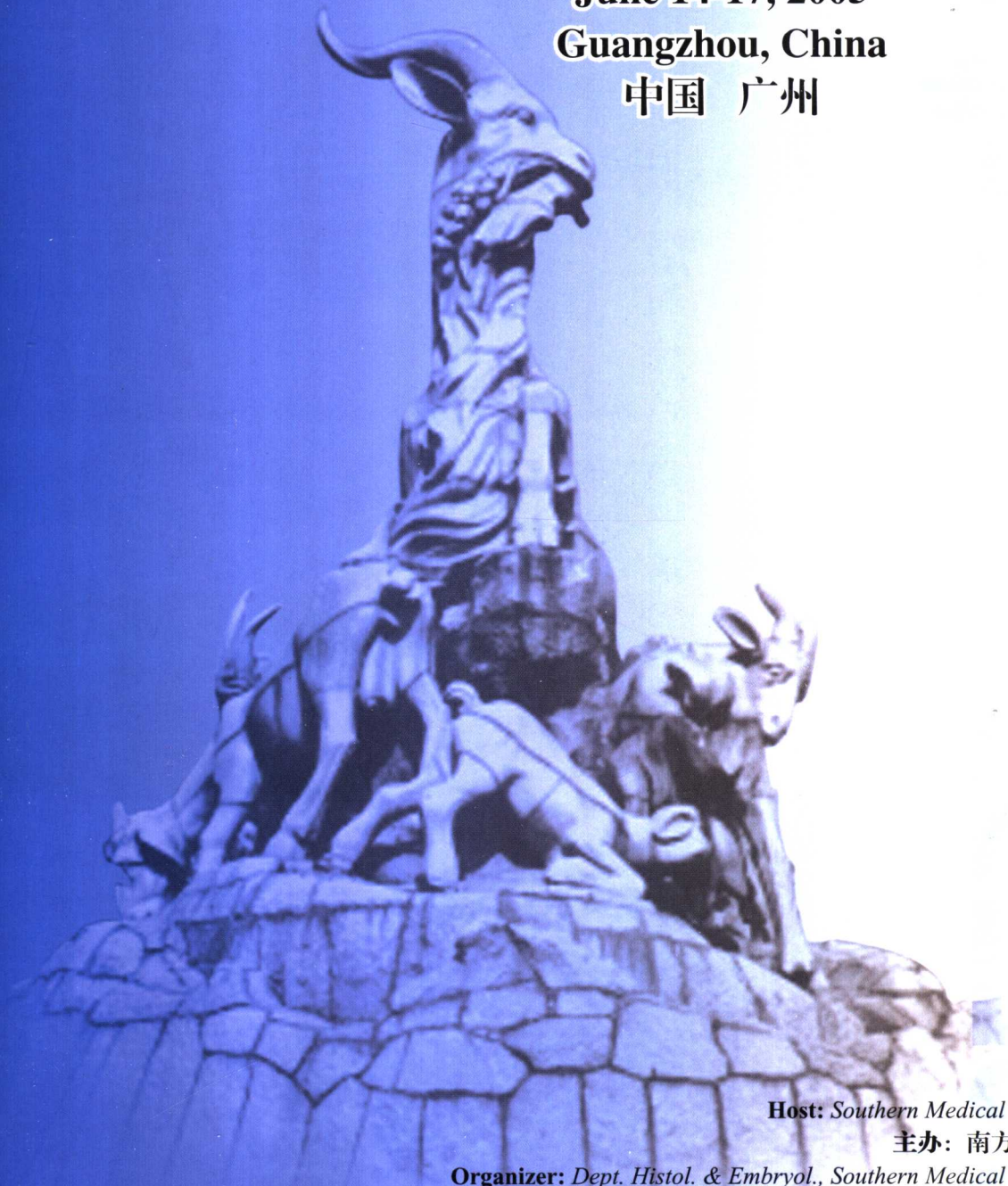

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6. Dr. *Thom Saunders*, University of Michigan, USA
7. Dr. *Biliang Zhang*, University of Massachusetts Medical School, USA
8. Dr. *Zhi Chen*, ZymoGenetics, USA
9. Dr. *Janice Parker-Thornburg*, University of Texas M. D. Anderson Cancer Center, USA
10. Dr. *Bertrand Pain*, Ecole Normale Supérieure de Lyon, France
11. Professor *Xigu Chen*, Department of Experimental Animal, Zhongshan Medical college, Sun Yat-sen University
12. Professor *Weiwang Gu*, Laboratory Animal Center, Southern Medical University, Member of the Chinese Academy of Sciences, China
13. Dr. *Xiao Yang*, Genetics of Development and Disease laboratory, Institute of Bioengineer, Academy of Military Medical Sciences, China
14. Dr. *Qi Zhou*, Research Group of Reproduction & Development, Institute of Zoology, Chinese Academy of Sciences
15. Dr. *L. Hiripi*, Department of Animal Biology, Agricultural Biotechnology Center, Godollo, Hungary
16. "VEGFR-3 signaling in tumor lymphangiogenesis and lymphatic metastasis"—Dr. Yulong He, Molecular / Cancer Biology Laboratory, Biomedicum Helsinki, University of Helsinki, Helsinki, Finland; Laboratory of Vascular and Cancer Biology, Model Animal Research Center, Nanjing University, Nanjing, China

17. **Dr. *Hua Tang*, Tianjin Life Science Ressearch Center, Tianjin Medical University**
18. **Dr. *Yaoting Gui*, University of Manitoba, Canada**
19. **Dr. *Shiliang Shen* – Stanford University, USA**
20. **Professor *Guoxiang Chen*, Shanghai Transgenic Research Center, Shanghai, China**
21. **Professor *Defu Zhang*, Animal Husbandry and Veterinary Research Institute, Shanghai Academy of Agricultural Sciences; Division of Animal Genetic Engineering, Shanghai Municipal Key Laboratory of Agri-Genetics and Breeding ; Shanghai, China**
22. **Professor Weiquan Liu, College of Biology, China Agricultural University, Beijing, China**

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首先，衷心地感谢您长期以来对《遗传学报》的关注及大力支持，也感谢您能在百忙之中阅读此函。

《遗传学报》是中国遗传学会和中国科学院遗传与发育生物学研究所主办、科学出版社出版的高级学术刊物，中文生物学、农学、农作物类核心期刊，已被美国化学文摘（CA）、生物学文摘（BA）、医学索引（IM）、俄罗斯文摘杂志（AJ）和《中国学术期刊文摘》等20余种国内外重要检索系统与数据库收录。《中国学术期刊综合引证报告》（2004版）据5716种统计源期刊2003年的主要文献计量指标，《遗传学报》总被引频次为**1454**,影响因子为**1.0224**，在“人类学与生物科学”期刊中排名**第二位**。《遗传学报》连续2年获得百种中国杰出学术期刊奖,2005年1月获得第三届国家期刊奖二等奖。

为了加快期刊的国际化步伐，扩大国际交流，《遗传学报》从2006年起改为英文版，并与国际著名出版社合作出版,扩大海外发行。从2005年5月1日起，**《遗传学报》同时接收中文或英文投稿，但中文投稿一旦录用后要求作者翻译成英文形式刊登。**

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《遗传学报》编辑部

2005年4月1日

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Thank you for your time and attention, and we are looking forward to your contribution.

Yours sincerely,

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Section 1 TRANSGENIC ANIMALS AND MAMMARY GLAND BIOREACTOR

（转基因动物与乳腺生物反应器）

0101 Construct Synthetic Gene Encoding Artificial Spider Dragline Silk Protein and its Expression in Milk of Transgenic Mice

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Abstract: Spider dragline silk is a natural protein with distinct mechanical properties such as high tensile strength and elasticity. Therefore it is useful in industrial, military and medical applications. Based on the known partial cDNA sequence of dragline silk protein, an artificial gene monomer, a 360bp sequence, was designed to encode an analog of dragline silk protein. DNA monomer sequence was polymerized to encode high molecular weight artificial spider dragline silk protein. Six tandem copies of monomer were cloned into pBC1 vector, a specific milk expression vector, and microinjected into the pronuclei of fertilized Kunming White eggs. Transgenic mice were screened by PCR and Southern blot which revealed that 10 mice (5♂, 5♀) among 58 mice were transgenic positive. The number of copies of the transgene ranged from 1 to 15, and the efficiency of microinjection was 17%. Milk of five F0 mice and eight F1 mice was analyzed by Western blot, and two F0 mice and seven F1 mice expressed recombinant dragline silk protein. In transgenic mice milk, a maximum of concentration of recombinant dragline silk protein was 11.7mg/L by radioimmunoassay.

Key words: spider, dragline silk, transgenic mice

Introduction

Spider dragline silk is a remarkable material with a combination of tensile strength and elasticity. Its tensile strength is about 4×109Nm-2 and its elasticity is about 35%, which makes it stronger and more resistive to breaking than any other common material, natural or artificial (Heslot, 1998; Vollrath, 2000; Hinman et al., 2000). Therefore, spider dragline silk could be useful in a variety of applications in industrial, military and medical purposes. Thus it has been the subject of intense studies.

Despite the number of potential applications for spider silk, the territorial behavior of spiders makes them impossible to farm in the same manner as silkworms and so there was no way to produce large amounts of natural spider silk. As a consequence, many research groups have attempted to use genetic engineering to produce synthetic spider silk protein (Wong Po Foo and Kaplan, 2002).

The dragline silk of spider Nephila clavipes is composed of two proteins, MaSp1 and MaSp2, whose partial amino acid sequences have already been determined (Xu and Lewis, 1990; Hinman and Lewis,

1992). The amino acid sequences of each MaSp can be generalized as sets of consensus motifs (Figure 1). The dragline silk proteins have four types of amino acid motifs:(1)Gly-Pro-Gly-Gln-Gln/Gly-Pro-Gly-Gln-Xaa (Xaa is Ala、Ser、Tyr or Val);(2)Gly-Gly-Xaa (Xaa is Ala、Tyr、Leu or Glu); (3) poly-Gla-Ala/poly-Ala; (4) Spacers (Hayashi et al., 1999; Hayashi and Lewis, 1998). Each motif adopts a specific secondary structure. The poly-Ala and poly-Gla-Ala regions adopt antiparallel β-sheet structures and are crystalline and highly oriented. These crystalline structures is a critical component in producing the observed the high tensile strength of spider silk (Thiel and Viney, 1996; Simmons et al., 1996; Hijirida et al., 1996). The Gly-Pro-Gly– Gln-Gln/Gly-Pro-Gly-Gln-Xaa regions adopt β- turn secondary structures, which are the mechanism behind the observed elasticity. The Gly-Gly- Xaa regions adopt helix secondary structures, which could serve as a transition or link between crystalline β-sheet regions and less rigid protein structures (Hutchinson and Thornton, 1994; Jelinski et al., 1999; van Beek et al., 2002; Parkhe et al., 1997; Wilson et al., 2000).

Some research groups have successfully expressed synthetic genes encoding dragline silk protein in microorganisms, e.g. yeast and bacterial (Lewis et al., 1996; Winkler et al., 1999; Arcidiacono et al., 1998; Fahnestock and Bedzyk, 1997; Fahnestock and Irwin, 1997; Prince et al., 1995). However, in microorganisms, dragline silk protein expression levels were very low and the products of expression were somewhat heterogeneous. The demand for large scale production of spider silk prompted studies into alternative hosts and some research groups recently expressed dragline silk synthetic genes encoding dragline silk protein in tobacco and potato, and dragline silk protein cDNA in mammalian cells (Scheller et al., 2001; Lazaris et al., 2002).

In this study we show that synthetic gene encoding the dragline silk protein can be efficiently and stably expressed in transgenic mice milk.

Materials and Methods

Construction of a gene for synthetic dragline silk protein

Based on the consensus nucleotide sequence from the highly repetitive dragline silk gene of spider Nephila clavipes, a gene monomer named X, was designed and synthesized. Monomer X was 360 base pairs and flanked by unique restriction nuclease sites (BglII and BamHI) with compatible ends (Figure 2(a)). It encoded a peptide of 118 amino acid residues (Figure 2(b)).

Monomer X was constructed as following steps: (1) Synthesizing four single-stranded oligonucleotides: A, B, C, and D, encoding partial fragments of MaSp1 and MaSp2. A and B, C and D, were designed to hybridize to each other in a complementary 24-base region at the 3'end of each fragment (shown in bold italic in Figure 2(a)). (2) After annealing, the remainder of the bases were filled in by Taq polymerase and produced the DNA fragment AB and CD. (3) After digestion with NheI, AB and CD were ligated and then cloned into the T-vector (Promega, USA) derived from the pGEM-5zf vector.

Vector pGEM-5zf containing monomer was double digested with BglII/NcoI (a restriction endonuclease site of pGEM-5zf), and the monomer was isolated by agarose gel electrophoresis and purified using a Qiagen Plasmid Purification Kit. The monomer was then cloned into the site between the BamHI and NcoI sites of pGEM-5zf containing monomer. The higher order multimer was produced using the same process. Finally, a six tandem copies of monomer X (X6) was produced and named pGEM5zf-X6.

Construction of DNA sequence for goat β-casein signal

The signal sequence constructing was carried out as the monomer X DNA sequence constructing. The goat β-casein signal peptide DNA sequence (Roberts et al., 1992), flanked by some restriction endonuclease sites (SalI, BglII and NsiI, indicated by bold italic), was synthesized and indicated by the underlined text, the forward sequence is 5'GTAAGATATCGTCGACTTCGAAATGAAGGTCCTCATCCTT GCCTGTCTGGTGGCTCTGGCCATTGCAAGATCTCCA 3', the reverse sequence is 5' CTTGTCGA ATTCGTCGACGGCGCGCCATGCATAACTTAATCCTTCTGATGGAGATCTTGCAATGGCCAG 3'. The signal peptide DNA was then cloned into T-vector (Takara, Japan), named pTV-S. Vector pGEM5zf-X6 was double digested with BglII/NsiI, and then the dragline silk gene was isolated by agarose gel electrophoresis and purified using a Qiagen Plasmid Purification Kit. The insert was then cloned into the site between the BglII and NsiI sites of pTV-S, producing the plasmid pTV-S-X6.

Construction of expression vector

Vector pTV-S-X6 was digested with SalI, the dragline silk gene was isolated by agarose gel electrophoresis and then cloned into the XhoI restriction site of pBC1 vector (Invitrogen, US), producing the pBC1-X6 expression vector.

Preparation the antiserum against dragline silkprotein

Based on the known partial amino acid sequence of spider Nephila clavipes dragline silk, a peptide of 19 amino acid residues was synthesized. The peptide sequence was: Cys-Gly-Pro-Gly-Gln-Gln-Gly-Pro-Gly-Gly-Tyr-Gly-Pro-Gly-Gln-Gln-Gly-Pro-Ser-NH2 (Fahnestock and Irwin, 1997). Antiserum was raised in rabbits against synthetic peptides conjugated to keyhole limpet hemocyanin.

Transgenic mice production

Vector pBC1-X6 was double digested with SalI and NotI. The insert was isolated by agarose gel electrophoresis and recovered by electroelution. To remove any contamination, product were spot dialyzed against 40ml TE (10mmol/L Tris, 0.1mmol/L EDTA, and pH7.4) for 30 minutes (VSWP02500 membrane, Millipore). Purified DNAs were diluted to 2-3ng/μl in TE buffer and microinjected into the pronuclei of fertilized eggs of Kunming White.

Genomic DNA was extracted from the tails of transgenic mice. A primer pair was then designed to screen for transgenic mice: upper primer: 5'- TCCCAGAATCTAAGCGATA-3' and downstream primer: 5'- ATGAGGACCTTCATTTCGAAGT-3'. PCR reactions using genomic DNA as template were performed under the following conditions: 30 cycles of 94°C for 1min, 58°C for 1min, and 72°C for 1min. The length of PCR product was 553bp. After PCR screening, transgenic mice were confirmed by Southern blot. The probe was created by α-32P labeling the X6 DNA sequence. 10μg of genomic DNA isolated from transgenic mice and wild-type mice was digested by BamHI. Hybridizations were at 65°C in Church solution (1%BSA, 7%SDS, 1mM EDTA, 0.5M Sodium phosphate, pH7.2). Final washes were in 2×SSC, 0.5×SDS at 65°C. Signal from the membrane was detected using a Phosphor Screen (Molecular Dynamics, US). Signal quantification was performed by scanning band density with software provided by Alphaimager 2200 (Alpha Imaging System, US). Copy numbers were determined by comparing the band density of dilutions of the pBC1-X6 plasmid digested with BamHI.

Northern blot

Total RNA was extracted from mammary tissue of mouse in lactation using Trizol (Invitrogen, US). The probes were created by α-32P labeling the X6 DNA and GAPDH partial cDNA sequence respectively. 30μg of total RNA was fractionated by formaldehyde gel electrophoresis. The RNA contained within the gel was then transferred to nylon membrane and immobilized. Hybridizations were performed at 68°C overnight in 5×SSC, 5×Denhart's solution (0.1%Ficoll, 0.1%BSA, 0.1%polyvinylpyrrolido- ne), 1%SDS and 100μg/ml salmon sperm DNA. Final washes were performed three times in 0.1×SSC/0.1%SDS at 68°C. Signal from the membrane was detected using a Phosphor Screen (Molecular Dynamics, US).

Western Blot and Quantification of dragline silk protein by Radioimmunoassay

The transgenic milk was collected at the 7th day of lactation. To remove fat fractions, milk samples were centrifuged at 4500r/min for 15 min. Then the lower layer liquids were adjusted to pH3.8-4.6 by 1N HCl to remove casein fraction. After electrophoresis in 10% SDS-PAGE gel, proteins were transferred to a nitrocellulose extra blotting membrane (Sartorius, Germany). Antiserums against synthetic peptides as described above and HRP-conjugated goat anti- (rabbit IgG) (Sino-American, China) were used to detect dragline silk protein.

For radioimmunoassay (RIA), the peptide, which was used to prepare against dragline silk protein antiserum, was radiolabeled with I125 by chloramine-T method. The standard curve was obtained by serial dilution of the unlabelled peptide. Competition binding was carried out at 4°C for overnight in PB buffer using polyclonal rabbit raised against dragline silk protein antiserum. Separation of bound from free was achieved by the PEG-double antibody method, followed by centrifugation. Then the radioactivity was measured by an automated γ counter.

Results

Production of transgenic mice

In total, 58 mice were born of which 10, (5 males and 5 females), were identified by PCR to contain the transgene construct (Figure 3). The lines of the positive mice were 3, 16, 22, 24, 36, 42, 43, 46, 55, and 58. Furthermore, these transgenic mice were confirmed by Southern blot (Figure 4). Efficiency of microinjection was about 17%, which is within the usual range of 5-20%. Copy numbers of transgene were also determined by Southern blot. The copy number of trangene ranged from 1 to 15. More than half of mice had low copies (1-3) of transgene, but high copy numbers (≥5) of transgene were also observed (Table 1).

All of founder mice were mated with wild-type mice except No.24 mouse, which died before being mated. F1 mice of the founder line were also identified by PCR. The results indicated that all of founders, with the exception of founder 16, transmitted the transgene to their progenies (Table 2).

Northern blot

The expression of the dragline silk gene was analyzed by Northern blot, by staining the membranes of the total RNA of F0 mice. The result of the Northern blot (Figure 5) indicated that 2 mice (founder 43 and 46) had positive bands. The dragline silk gene mRNA molecular weight of founder 43 was same as that of founder 46 mice. Compared to the result of GAPDH Northern blot, the results of dragline silk gene Northern blot showed the mRNA molecular weight of the two mice dragline silk genes was bigger than of the GAPDH mRNA, while the band density of the dragline silk gene mRNA was lower than that