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Production of Transgenic Goat Encoding Spider Dragline Silk

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Key words
Spider silk-like protein, Albas cashmere goat, Somatic cell nuclear transfer, Tensile strength, Goat wool, Biomaterial

ABSTRACT

Spider silk, known for its remarkable properties and numerous advantages, shows promise in various fields as a fibrous biomaterial. However, research on obtaining transgenic goats through the artificial synthesis of spider silk protein is lacking. In this study, DNA sequence of the spider dragline protein monomer S, and the spidroin gene 4S multimer was obtained through doubling dimerization strategies. Subsequently, the vector containing synthetic spider silk 4S protein was introduced into the fibroblasts of Albas cashmere goats. Finally, embryos were constructed using somatic cell nuclear transfer (SCNT), and transgenic goats were obtained. The results show that a total of 2605 oocytes were retrieved from goats, of which 1469 reached maturity after 18h, produced maturation rate of 56.39%. 752 embryos underwent nuclear transfer, produced a fusion rate of 51.19%. In the fused embryos, 66.22% (498) underwent cleavage and subsequently transferred. The wool derived from transgenic goat demonstrated higher tensile strength and elongation in the foreleg and hindleg regions when compared to the nontransgenic goat wool ($P<0.05$). In conclusion, the expression of the spidroin gene 4S was achieved in goats. This study will hopefully improve the tensile strength of goat wool and provide a reference for the expression of artificial spider silk.

INTRODUCTION

Spider dragline silk has an excellent combination of strength, extensibility, good biocompatibility and biodegradability. It also has broad application prospects in medical treatment (construction of nerve guide catheters and medical sutures) and the textile industry (biopolymer materials). Spider dragline silk protein has attracted increased attention due to its good mechanical properties (Kono *et al*., 2021). The main components of dragline silk are two highly conserved spidroins, major ampullate silk protein (MaSp1) and MaSp2. The two spidroins possess two notable structural features: Both contain a number of repetitive motifs in the primary structure (Xi *et al*., 2021). According to research findings, MaSp1 and MaSp2 proteins in spider silk drawing, especially the presence of proline in MaSp2, can be used to drive characteristics to address a humid environment (Liu *et al*., 2019). Recently, some researchers found that MaSp3 may be one of the main paralogs of MaSp1 and MaSp2, and it contributes to the increase in mechanical properties. MaSp3, similar to MaSp1 and MaSp2, plays an important role in pH-sensitive dimerization of the N-terminal domain and contributes to silk formation (Kono *et al*., 2021). Thus, MaSps are an important component of spider silk, and their modification

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Abbreviations
SCNT, somatic cell nuclear transfer; KAPs, keratin-associated proteins; MaSp1 and MaSp2, major ampullate spidroins; PBS, phosphate-buffered saline; FBP, first polar body; COCs, cumulus-oocyte complexes; SD, standard deviation.

will help reproduce the advantages of spider silk. Since the living habits of spiders severely restrict the development and utilization of natural spider silk, biotechnology is used to help produce spider silk in different hosts and recombine to obtain spider silk in different forms and play a major role (Bakhshandeh *et al*., 2021).

In recent years, many reports have shown that spider silk protein can be produced by *Escherichia coli* (Bhattacharyya *et al*., 2021), yeast (Teulé *et al*., 2009), eukaryotes (Karatzas *et al*., 2005) and animals (Xu *et al*., 2007). With the development of technology, progress in the genetic engineering of spider silks has advanced spider silk research and applications. However, these methods still have shortcomings, such as unstable expression systems, low efficiency, lack of molecular orientation of recombinant proteins, and inability to simulate the fibrosis process of spider silk. Scientists have attempted to improve the expression of exogenous genes by optimizing genetic information, adjusting codon preferences, and looking for more efficient expression vectors to obtain high-yield, high-molecular-weight recombinant spider silk proteins (Xia *et al*., 2010). Studies have reported a mammary-specific high-efficiency expression vector encoding artificial spider pulling silk protein and expressed it in transgenic mice (Xu *et al*., 2007). Previous studies have attempted to obtain large quantities of spider silk protein by using transgenic goat milk, and the transgenic goats produced fairly low concentrations of silk proteins in their milk (Center, 2010; Williams, 2003). Somatic cell nuclear transfer (SCNT) was used to express spidroin in sheep, which are rich in wool products, thereby increasing the toughness of wool. Unfortunately, no offspring individuals were obtained, and further research is still required (Li *et al*., 2020).

The Inner Mongolia Albas cashmere goat is renowned for its high cashmere yield and good quality (Dai *et al*., 2020; Gong *et al*., 2022). However, because wool fibers are inherently weak, fiber breakage during spinning and weaving processes, which are commonly carried out under tension, is a major problem. Fiber breakages reduce the production efficiency, create fabric defects, and generate substantial amounts of fiber and fabric wastes (Prins, 2009). Increasing the strength of the fibers can not only solve these issues but also allow spinning finer yarns from the same fibers, substantially increasing their value. Spider silks have excellent fiber properties, although they are available in significantly lower quantities due to the cannibalistic nature of spiders and the difficulties of domestication (Mu *et al*., 2020).

In this study, an artificially synthesized fragment was transfected into Albas cashmere goat fibroblasts to obtain transgenic goats expressing the spider silk protein-encoding gene in goat wool through SCNT. In summary, this study aims to improve the toughness of wool and reduce the losses caused by insufficient toughness during artificial textile manufacturing processes.

MATERIALS AND METHODS

Plasmids

The 561 bp spider dragline silk protein-encoding gene S was synthesized by Sangon Biotech according to the sequences of Spidroin1 and Spidroin 2 of spider (*Nephila clavipes*) published in GenBank (AY555585 and AH015065). After synthesis, S was ligated into PUC57 (Thermo Scientific) to construct PUC57-S and flanked by four restriction enzyme sites: BamHI and NaeI, SmaI and NotI (TaKaRa). For dimerization of S, PUC57-S was digested by NaeI and NotI, and S was extracted by a Gel Extraction Mini Kit (TaKaRa). Then, S was ligated into PUC57-S, which had been digested by SmaI and NotI to yield PUC57-2S. NaeI and SmaI are isoschizomers, and the cohesive terminus sequence digested by NaeI can ligate to the cohesive terminus of SmaI to form 2S. However, the restriction enzyme site was destroyed after ligation of the cohesive terminus of NaeI, and SmaI was not used again. Then, PUC57-4S was constructed by restriction enzyme digestion and ligation of PUC57-2S in the same way.

The Kap6.1 promoter (Supplementary Fig. 1) was amplified by PCR with sheep genomic DNA as a template, and primers were designed based on the sequence available on NCBI. The original CMV promoter of pEGFP-N1 was replaced by the amplified Kap6.1 promoter to construct pKap-EGFP. The 4S fragment was cloned from pUC57-4S and inserted into pKap-EGFP (supplied by the author's laboratory), and 4S was inserted downstream of the specific keratin Kap6.1 promoter and enhanced green fluorescent protein to construct pKap-EGFP-4S. Subsequently, goat fetal skin fibroblasts were transfected with the modified plasmid to assess the activity of the promoter.

PCR and RT–PCR

In this study, PCR and RT–PCR were employed to identify transgenic cell lines placentas and umbilical cord. Genomic DNA and RNA were extracted from the cell lines and umbilical cords using specific extraction kits, including the cell genomic DNA extraction reagent (TianGen), TRIzol RNA extraction reagent, and RNA reverse transcription kit (TaKaRa). PCR is performed using DNA as a template, while RT-PCR utilizes cDNA as a template. The gDNA remover was employed to mitigate any DNA contamination during the RT-PCR process.

The sequences of primers used were as follows: GADPH F3: GTCGGAGTGAACGGATTT; R3: TCACGCCCATCACAAAC. Keratin Kap6.1 promoter:

F1: GACGTCCTGAGCGACTTCACTTT; R1: AAGCTTTGTTGCTTGTTGAGGTTG. Spider silk-like protein-encoding gene: F2: CGTAAACGGCCACAAGTTCAGCGTG; R2: AGTTCACCTTGATGCCGTTCPCR. Reverse transcription system:5×Prime script buffer (for real time), 2μL; prime script RT enzyme mixI, 0.5μL; Oligo dT Primer (50μM), 0.5μL; Random 6 mers (100μM), 0.5μL; Total RNA, 1μL-2μL; RNase Free d3H2O add to 10 μL.

Cell culture and transfection

Fibroblasts were isolated aseptically from Albas cashmere goat fetuses. In brief, the fetus was placed into sterile phosphate-buffered saline (PBS) and 75% alcohol for 10-30 s, removed and washed 3 times with a beaker containing an appropriate amount of sterile PBS. Then, the tissue was cut into small pieces of 1-2 mm^3 with sterile scissors and transferred to a Carlisle flask for cell culture. On the day before transfection, the 6-well plate was coated with 2 $\times 10^5$ cells/L, and the confluence rate of cells was required to reach 70%-90%. Lipofectamine 2000 was used for transfection. The transfected cells were selected with 500 μg/mL G418 (Sigma). The G418-resistant colonies were pooled and used to clone transgenic embryos by nuclear transfer.

In vitro *maturation of goat oocytes*

Goat ovaries were obtained from a slaughterhouse in Hohhot, China. With a 10 mL syringe equipped with an 8# needle, follicles with a diameter of 2-4 mm on the surface of the ovaries were aspirated to collect 2-3 mL of oocyte collection solution (consisting of M199, $NaHCO_3$, 1 μg/mL heparin, and 2 mg/mL BSA). The collected follicular fluid was then transferred to a 60 mm petri dish for further use. Subsequently, the collected cumulus oocyte complexes (COCs) were subjected to 3 washes in a prebalanced maturation solution. The maturation solution comprised M199 with 10% FBS, 10 μg/mL FSH, 20 μg/mL LH, 1.5 μg/mL 17-ß E2, 0.38 mmol/L sodium pyruvate, 100 IU/mL penicillin, and 100 IU/mL streptomycin. The COCs were cultured under specific conditions of 38.5 °C, saturated humidity, and 5% CO_2 concentration. After 18 h of culture, the cumulus cells were removed, and the naked oocytes that had discharged the first polar body (FBP) were selected for nuclear transplantation.

Enucleation and microinjection

The oocytes chosen for cytoplasmic receptor treatment were subjected to H-M199, which refers to M199 supplemented with 25 mmol/L Hepes, for a duration of 5 min. Subsequently, the oocytes underwent a series of preparative steps before microinjection. The individual transfected cells were transferred into the perivitelline space of the enucleated oocytes. The constructed recombinant embryos were placed in culture drops and cultured for approximately 20 min prior to fusion.

Fusion and activation

The reconstructed embryos were immersed in the fusion solution (0.3 M mannitol, 0.5 M HEPES, 0.05 mM magnesium acetate, 0.05% BSA, and 0.1 mM calcium acetate) for a duration of 2 min, followed by transfer into the fusion tank. Two electric shocks were administered, each lasting 10 μs, with a voltage of 130 V/mm. After completion of 30 fusions in each group, the embryos were washed 3 times with H-M199 and returned to the maturation solution. Subsequently, the embryos were incubated in a CO_2 incubator for 20 min to facilitate their recovery.

Embryo culture

The genetically modified embryos were cultured in maturation medium for a period of 4 h. After the specified duration, they underwent a brief activation for 5 min using H-M199 with 5 μM ionomycin and 10% FBS. Following activation, the embryos were transferred into SOFaa medium containing 2 mM 6-DMAP and cultured for an additional four h. Finally, the reconstructed embryos were placed into droplets of SOFaa culture medium, with each droplet containing 20-30 embryos.

Embryo transfer and pregnancy evaluation

Before transplantation, the recipient goats, which were cashmere goats purchased from Arbas in Ordos, underwent estrus synchronization to prepare for the procedure. Embryos in the early stages of development were transferred into the ampulla of the oviduct, while the morula and blastocyst were transferred into the uterine horn. For confirmation of pregnancy, a trans-rectal ultrasound was performed 60 days after embryo transfer.

Toughness detection of transgenic goat wool

The tensile force and elongation of transgenic goat wool and nontransgenic goat wool were assessed using an electronic tensile testing machine (DSC07067, Changzhou Second Textile Instrument Factory). First, the instrument was correctly positioned, and it underwent further adjustments to ensure its levelness. The testing method was selected based on the specific requirements, and the gauge length was determined according to the wool characteristics. For fabrics with a breaking elongation of 75% or less, the gauge length was set at 200 mm ± 1 mm. The spacing was 10 mm, the pretension was 0.1 cN, and the tensile speed was 20 mm/min. The test was initiated by clicking the "Test" button, which stretched the sample until it reached the point of rupture. The resulting data,

including the breaking force (measured in N) and the elongation at break (measured in mm), were carefully examined and recorded for analysis.

Statistical analysis

The data are presented as the mean ± standard deviation (SD). The wool toughness testing data, including tensile force and elongation, were analyzed using nonparametric tests. $P < 0.05$ was considered statistically significant in detecting differences between the groups.

RESULTS

Vector construction and transfected cells

To enhance the resemblance of artificially synthesized spider silk to natural silk, we subjected S to double dimerization to synthesize 4S. The pKap-EGFP-4S construct was created using the sequence of the spider-like dragline protein 4S (Fig. 1A). Fibroblasts were transfected with pKap-EGFP-4S, and monoclonal cells were selected to generate transgenic embryos (Supplementary Fig. 2). To confirm the presence of the transgene in the positive cell line, we performed PCR using goat monoclonal cell line genomic DNA as the template. The results revealed that 5 clones exhibited present of the spider-like dragline protein 4S (Fig. 1B). Furthermore, RT–PCR analysis (Fig. 1C) provided additional evidence that the foreign gene had been successfully transferred into cells.

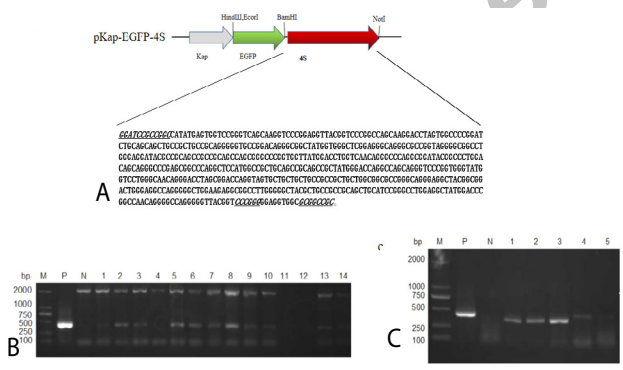

Fig. 1. Identification of the goat fibroblast line containing the spider silk-like protein-encoding gene. A, Construction of a vector containing spider silk-like protein (the markings indicated by diagonal lines represent specific enzymatic cleavage sites). B, PCR identification of goat monoclonal cells. Note: M, DL2000; P, positive control (plasmid); N, negative control (non-transgenic cells); 1-14, The PCR products of different cell lines containing transgenic spider silk fragments target band (431 bp). C, Identification of goat monoclonal cells by RT-PCR. 1-5, The RT-PCR products of different cell lines containing transgenic spider silk fragments, target band (431 bp).

Development of reconstructed embryos

A total of 2605 goat oocytes were collected, out of which 1469 oocytes underwent extrusion of the FPB after 18 h, resulting in a maturation rate of 56.39% (1469/2605). After introduction of transgenic cells into enucleated oocyte cytoplasts, a total of 759 reconstructed oocytes were obtained. The fusion and cleavage development rates were 51.19% and 66.22%, respectively (Table I).

Table I. The data regarding the production rates of reconstructed embryo development.

	Number	Proportion
Extracted oocyte	2605	
Mature oocyte	1469	56.39%
Fused oocyte	752	51.19%
Cleavage oocyte	498	66.22%

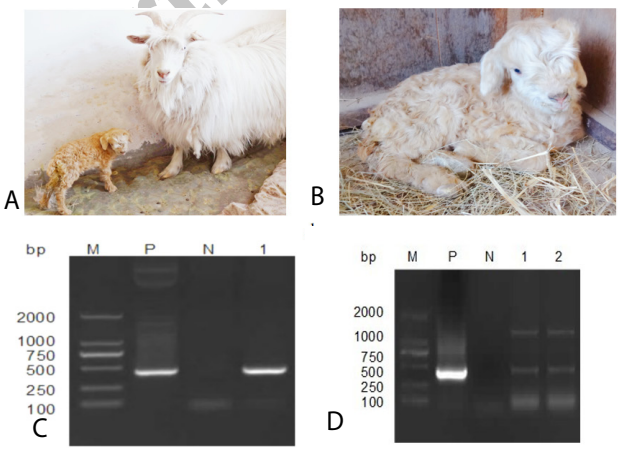

Fig. 2. Birth and identification of a transgenic goat. A, female goat and kid goat; B, kid goat; C, PCR identification of transgenic goat placenta. M, DL 2000; P, positive control (plasmid); N, negative control (non-transgenic cells); 1, The PCR product of placental tissue; D, RT–PCR identification of transgenic goat placenta. M, DL 2000; P, positive control; N, negative control; 1, 2, respectively represent the. RT–PCR products of placental tissue and umbilical cord.

Birth and identification of transgenic goats

A total of 46 cleavage embryos were transplanted into 23 surrogate ewes. B-ultrasound monitoring and observation of mammary gland development revealed that two surrogate ewes had a normal pregnancy in the second trimester, resulting in the birth of a cashmere goat lamb 5 months later (Fig. 2A, B). The pregnancy rate of SCNT was 8.6%. At the time of delivery, the placental tissue was preserved, and the genome was extracted for PCR

Table II. The analysis of the toughness of wool (mean±SD).

Sampling position	Measured number (wool)	Tension (cN)		Elongation (mm)	
		Transgenic goat	Nontransgenic goat	Transgenic goat	Nontransgenic goat
Foreleg	200	20.59±3.96**	18.06±4.76	4.63±0.96**	3.72±0.93
Hind leg	200	22.57±9.11**	20.01±7.87	3.96±0.83**	3.66±1.03
Waist	200	20.20±7.81	19.94±5.83	4.06±0.63	3.85±1.13

The breaking tensile force and elongation of wool between nontransgenic (n=6) and transgenic goats (n=1) were significantly different (P<0.05).

detection (Fig. 2C). The electrophoretic diagram showed an amplified fragment of 431 bp in the first lane, which was consistent with the positive control, indicating successful integration of the gene of interest into the goat genome. Additionally, RT–PCR amplification was performed on transgenic goat placental RNA (Fig. 2D), confirming the expression of the transferred foreign gene within the goat genome.

Toughness detection of transgenic goat wool

For analysis of the tensile strength and elongation of goat wool, a total of 200 wool samples were randomly collected from various locations on both transgenic and nontransgenic goats of the same age and strain. These locations included the foreleg, hind leg, and waist (Table II). In addition to the waist region, the tensile strength and elongation of wool obtained from transgenic goats were significantly superior to those observed in nontransgenic goats (P <0.05).

DISCUSSION

Spider filament protein fibers exhibit distinct secondary structures. The ordered crystalline region is predominantly composed of polyalanine β-sheets, while the glycine-rich region primarily forms an α-helix of 3_{10} structure (Wang *et al*., 2019). Lewis *et al*. (1996), Rising *et al*. (2010), Candelas *et al*. (1990), and Hinman and Lewis (1992), reported that the spider dragline silk protein was encoded by MaSp1 and MaSp2. Given its remarkable elasticity and strength, we synthesized an artificial spider dragline protein sequence, denoted as S, based on the Spidroin1 and Spidroin2 proteins derived from the spider species *N. clavipes*. The incorporation of these two structural components gives rise to the extraordinary mechanical characteristics observed in spider silk. First, we employed a ratio of 3:2 for the MaSp1 and MaSp2 proteins, which bear resemblance to natural spider silk. Second, we refined the synthesized genes to exhibit a stronger preference for mammalian codons, thereby facilitating enhanced expression in eukaryotic cells. Finally, we artificially assembled the monomer S of the synthetic spider silk protein through double dimerization processes, resulting in the formation of a tetrameric spider silk protein. We exerted utmost effort in designing the spider silk protein to promote improved expression.

Goat wool is primarily composed of keratin and keratin-associated proteins (KAPs), which are intertwined to form a network structure. Studies have confirmed that sheep Kap6.1 is a high glycine/tyrosine keratin-binding protein, and cDNA in situ hybridization experiments have shown that its expression is mainly confined to cortical cells in hair follicles (Yang *et al*., 2014). Keratin-associated proteins are important structural components of fibers that are predominantly present in the ortho-cortex (Zhao *et al*., 2021). However, the results from this study demonstrate its expression in the placenta. According to the literature records, keratin is distributed in the endometrium during early goat pregnancy, and its content changes with the passage of time (Khong *et al*., 1986). During embryonic development in mice, abundant expression of keratin 8 in the mesoderm may play a crucial role in the expansion of extraembryonic structures (Khong *et al*., 1986). The wool test results showed that transgenic goats were significantly superior to nontransgenic goats in the foreleg and hind leg. This finding does not preclude the possibility that the results are due to individual variations. Research has reported that female Angora goats (*Capra aegagrus hircus*) aged 11-12 months have a breaking force of 18 cN. However, the specific location was not indicated in the data (Jankowska *et al*., 2019), although the value closely resembles the values obtained from the foreleg of nongenetically modified goats in this study. Another study revealed that the average breaking strength of Inner Mongolian Sunitt sheep at 12 months of age was 6.86 cN (Mingxiang-Sun *et al*., 2023). Research has also indicated that wool characteristics differ between the early-developing shoulder region and the late-developing leg region (Thomasset, 1938). Differences in tensile strength and elongation between the wool fibers of the waist and leg regions may be attributed to developmental factors. To date, there has been limited research on goat lamb wool. However, the present study is subject to limitations due to the generation of a transgenic goat.

The use of mesenchymal stem cells as nuclear donors guarantees the successful generation of transgenic animals (Ramiro *et al*., 2018). However, mesenchymal stem cells tend to undergo senescence at early passages. After 7-10 passages, there can be notable changes in cell morphology, telomere shortening, and proliferative arrest, which can make it challenging to edit and clone the cell lines (Vidal *et al*., 2012). Therefore, this issue could be a potential reason for the slightly lower rate compared to other studies. In this study, the pregnancy rate was 8.6%. In a study involving cattle using the SCNT method, the pregnancy rates of SCNT-produced fetuses were 13.04% on gestational Day 180 (Gao *et al*., 2019).

CONCLUSION

In conclusion, the successful synthesis of the spider-like dragline silk protein-encoding gene monomer S was achieved, with the ability to be artificially duplicated. The integration of the spider-like dragline silk protein-encoding gene into the goat genome via SCNT was successfully accomplished. One transgenic kid goat wool exhibited enhanced strength and elongation in the foreleg and hind leg regions compared to that of six ordinary goats of the same breed, this is also the limitation of this study. This report lays the foundation to produce cloned goats capable of expressing spider dragline silk protein in their wool through SCNT technology, opening new possibilities for the utilization of spider silk proteins.

DECLARATIONS

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IRB approval

This work was approved by the Agricultural University Biomedical Research Ethics Approval [2019]039.

Ethical statement

The study strictly adhere to laboratory operating procedures and ensure compliance with animal welfare standards, and follow the “3R” principle. The produced animals were used only for scientific research and die naturally.

Supplementary material

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20240816031913

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Production of Transgenic Goat Encoding Spider Dragline Silk

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GACGTCCTGAGCGACTTCACTTTCATGTATCACTTTCATGCATTGGAGAAGGAAA
TGGCAACGCACTCCAGTGTTCTTGCCTGGAGAATCCCAGGGCTGGGGGAGCCTGG
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TAAGAGGAATCTAAACTGAGATGTCAACACAGCAAGCCTGCTGAATTTCTCTGAG
GTTTCATCTTTGGTTGTGAACAACAAGCTAATTAGTCCAGTCATAAAGTTAGCCA
ATGGCATGAAGGTGTGGTGGGTCACACCCACACTGAGAGCATACAAAAGGCCCTC
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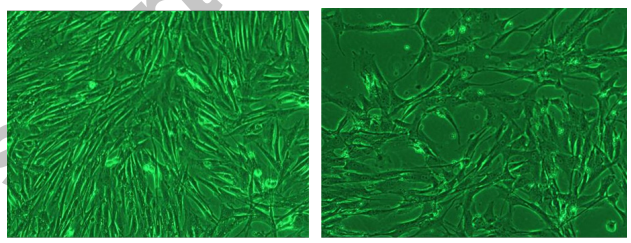
GAACAACCTAAGCTT

Supplementary Fig. 1. The sequence of the KAP6.1 promoter.



Supplementary Fig. 2. The morphology of non-transgenic goat fibroblasts (Left) and transgenic goat fibroblasts. The non-transgenic goat fibroblasts were F3 cells after resuscitation (100 ×); the transgenic goat fibroblasts g1-14 F10 cells were selected (100 ×).

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