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Cloning of *GH*, *IGF-I* and *SST* Genes of Anhui White Goat for Transgenic Application

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Abstract: The objective of this study was to clone Growth Hormone (*GH*), Insulin-like Growth Factor-I (*IGF-I*) and Somatostatin (*SST*) genes of Anhui white goat for transgenic application. The total RNAs of Anhui white goat was extracted and the target genes *GH*, *IGF-I* and *SST* were amplified with PCR technique, then the cDNA fragments were cloned into the eukaryotic expression vector pEGFP-N1 to construct transgenic vectors. The recombinant plasmids were transfected into the fibroblasts of Anhui white goat by liposome. The expression of the transferred *GH*, *IGF-I* and *SST* genes in the transgenic fibroblasts was detected by Reverse Transcription Polymerase Chain Reaction (RT-PCR). The stable transfection clones were screened by G418 resistance and detected by PCR amplification. The three genes were successfully cloned, their transgenic expression vectors were constructed and their stable transfection fibroblast clones were obtained. The constructed transgenic expression vectors and stable transfection fibroblast clones of these genes provide alternative materials for breeding transgenic goats, especially Anhui white goat.

Key words: Anhui white goat, GH, IGF-I, SST, cloning, transgenic vector

INTRODUCTION

Anhui white goat is an important local variety of China that are extensively bred in the area of middle and lower reaches of Yangtze River. The main producing area of this goat is in Anhui and Jiangsu Provinces. After a long period of selective breeding, this goat has several excellent traits including high adaptability to roughages, resistance to high temperature and humidity, high reproduction rate and high-quality skin (Chen *et al*., 2009). In 2009, Anhui white goat was selected into provincial genetic resources protection list of livestock and poultry (Wang, 2009).

At present, this goat is mainly used to produce skins and material hairs for Chinese brushes in its main producing area. For example, Haimen of Jiangsu Province, one of major producing area of white goats is one of major countries that produces Chinese Goatskins. On the other hand, this goat is also famous for its early maturity, high fecundity and the excellent quality of meat. These traits are useful for breeding it into a meat goat. However, this goat has a slow growth rate, it is unfavorable for this application. In recent years, the two-way and three-way cross-breedings have been used to improve the growth rate of Anhui white goat (Ren *et al*., 2003), however, the cross-breeding seriously reduced the meat taste of this goat. Therefore, the new breeding way to improve the growth rate but retain its natural meat taste through the lowest genetic disturbance is worthy to explore such as transgenic breeding.

The target genes are necessary materials for the transgenic breeding of goat. To improve the growth rate, two genes coding hormones regulating animal growth have been commonly used in transgenic breeding of animals. Growth Hormone (GH), a single chain protein secreted by the anterior pituitary has the physiological function of promoting the vertebrate organism growth and development and accelerating the protein synthesis and promoting the lipid degradation (Miller and Eberhardt, 1983). GH has been shown to significantly increase the growth rate of livestock, accelerate the muscle growth and reduce fat synthesis. Over the past few decades, the researchers have achieved good results of improving production performance and growth rate of animals by transferring the *GH* gene. For example, the genetically modified pig was obtained by introducing human *GH* gene into the fertilized eggs of pig and the transgenic pig had improved growth rate and feed utilization efficiency compared with the non-transgenic littermates (Pursel *et al*., 1985). Two pig lines were produced by introducing bovine growth hormone genes into pigs and their growth rate increased by 11-14% (Pursel *et al*., 1989). The growth rate and wool production of transgenic

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sheep introduced with sheep growth hormone gene were significantly improved than the control group (Adams and Briegel, 2005).

Insulin-like Growth Factor (IGF), a single-chain polypeptide substance is composed by two single-chain polypeptide growth factors IGF-I and IGF-II. IGF-I is named for its structure similar to insulin, it can promote the growth and metabolism of a variety of tissue cells by mediating GH and plays a very important role in the growth process of the animals (Rinderknecht and Humkel, 1976). High expression of IGF-I in skeletal muscle promotes proliferation, growth and repair of muscle cells (Florini *et al*., 1996). Specific expression of IGF-I in muscle can significantly increase body weight and muscle weight of the transgenic mice (Shavlakadze *et al*., 2005).

Although, the *GH* and *IGF-I* genes of other species have been cloned and used in gene transfection yet in the opinion, they should not be rashly used to this goat in order to avoid potential genetic pollution. It is better to use the genes of this goat itself in its transgenic improvement. For this purpose, the growth-related genes should be cloned from the tissues of this goat itself and used to transgenic breeding of this goat. For this purpose, in this study, researchers cloned the genes of *GH* and *IGF-I* from tissues of this goat and constructed their eukaryotic expression vectors. To provide a more direct and mature genetic materials for transgenic breeding of Anhui white goat, researchers also transfected fibroblasts of Anhui white goat with two genes and screened the stable transfection clones in this study. Researchers also performed similar studies to another growth-related gene, Somatostatin (*SST*) for exploring its potential application in transgenic breeding of Anhui white goat.

MATERIALS AND METHODS

Primers for gene cloning: The PCR primers were synthesized by Sangon Biotech (Shanghai, China). The sequences of primers were as follows: GH-forward: 5'-CTG CAG GTC GAC TGA CAG CTC ACC AAC TAT GAT GG-3'; GH-reverse: 5'-ACC CGG GGA TCC CAG ATG GCT GGC AAA TAG AAG G-3'. IGF-I-forward: 5'-ACG CGT CGA CAG CAA TGG GAA AAA TCA GCA-3'; IGF-I-reverse: 5'-CGC GGA TCC GGA AGG TCT TCA TAC ATT CTG TAG T-3'. SST-forward: 5'-ATG CTG TCC TGC CGC CTC C-3'; SST-reverse: 5'-AGT TAA CAG GAT GTG AA A GTC TTC CAG A-3'. The italics letters of the reverse primers of GH and IGF-I represent an introduced point mutation of the stop codon.

Preparation of cDNA with RT-PCR: The pituitary, liver and pancreas tissues of Anhui white goat were obtained from Feidong goat farm (Hefei, China). Total RNA was extracted from the pituitary, liver and pancreas tissues of Anhui white goat with Trizol Reagent (Invitrogen, USA) and was reversely transcribed using the reverse transcription kit (Fermentas, USA). PCR amplification were performed as followings: 95°C for 10 min; 40 cycles of 95°C for 30 sec, 67°C (GH)/67°C (IGF-I)/64°C (SST) for 30 sec and 72°C for 1 min; 72°C for 5 min. Final amplification products were visualized on 2% agarose gels and purified by Gel purification kit (Axygen, USA).

T-A cloning of target fragments: The plasmids of pGEM-T, pMD18-T and pUCm-T were obtained from Sangon Biotech (Shanghai, China). The ligation system consisted of 7 μL of target fragments, 1 μL of T-Vector, 1 μL of T4 DNA ligase (Promega, USA), 1 μL of 10×T4 DNA ligase buffer and were incubated overnight at 16°C. The ligation products were transformed into 100 μL of *E. coli* DH5α competent cells (TIANGEN Limited., Beijing, China) and the positive recombinant plasmids (pGEM-T-Easy-GH, pMD18-T-IGF-I and pUCm-T-SST) were identified by ampicillin resistance, colony PCR and restriction enzyme digestion (Fermentas, USA) and then sequenced (Sangon Biotech, Shanghai, China).

Construction of recombinant eukaryotic expression vector: The double-enzyme digestions of plasmids were performed in two groups. The first group included pGEM-T-Easy-GH, pMD18-T-IGF-I and pEGFP-N1 (Clontech, USA), they were digested by BamHI and SalI. The second group included pUCm-T-SST and pEGFP-N1 plasmids, they were digested by XhoI and KpnI. The products of enzyme digestion were fractioned using 1% agarose gel electrophoresis and the target DNA fragments were recovered using Gel purification kit (Axygen, USA). The purified target DNA fragments were ligated into linearized pEGFP-N1 using the following reaction: 7 μL purified target DNA fragments, 1 μL linearized vector pEGFP-N1, 1 μL T4 DNA ligase (Promega, USA), 1 μL 10×T4 DNA ligase buffer. The reactions were incubated overnight at 16°C. The *E. coli* DH5α competent cells (100 μL) were transformed with the ligation products. The positive clones were screened by Kanamycin resistance and confirmed by colony PCR. The recombinant plasmids, pEGFP-N1-GH, pEGFP-N1-IGF-I and pEGFP-N1-SST were extracted from the positive clones and detected with double-enzyme digestion and sequencing (GenScript, Nanjing, China).

Transfection of Anhui white goat fibroblasts: The fetal fibroblasts of Anhui white goat were prepared by Anhui

Agricultural University. The cells were cultured in DMEM (Invitrogen, USA) to the density of 4×10^5 (about 80% confluence) in 60 mm petri dish and transfected with a mixture consisting of 10 μL (8 μg) plasmid DNA (pEGFP-N1-GH or pEGFP-N1-IGF-I or pEGFP-N1-SST) and 20 μL (20 μg) Lipofectamine™ 2000 (Invitrogen, USA) for 5 h at 37°C. The control group was transfected with pEGFP-N1 and blank control group was transfected with no plasmid. After transfection, the media containing plasmid and liposome was discarded and cells were cultured in fresh DMEM.

Screening of positive cell clones with G418: After the cells were cultured for 19 h, the cells were observed with IX51 fluorescence microscopy (Olympus, Japan) for detecting the transient expression of genes. The cells were collected by trypsinization and transferred into 100 mm petri dish and cultured in DMEM. When the confluence was up to about 80%, the cells were cultured in the media containing G418 at the concentration of 600 μg mL^{-1} for 7 days and the media containing G418 at the concentration of 300 μg mL^{-1} for 7 days. During this time, the culture media containing G418 were replaced per 3-4 days. Then, the positive clones were picked and successively subcultured in 48-well plates, 12-well plates, 60 mm petri dish and 100 mm petri dish. During the series of subculturing, cells were always cultured in the media containing G418 at the concentration of 200 μg mL^{-1}. In each subculture, the cells in the center of clone were collected and passaged for purifying clones.

Detection of target gene's expression by RT-PCR: To detect the transcription of target genes in the transgenic fibroblasts, a pair of pEGFP-N1 vector-specific PCR primers was designed. The sequences of primers were 5'-CAG AGC TGG TTT AGT GAA CCG TCA G-3' and 5'-TCC TCG CCC TTG CTC ACC A-3'. The primers were synthesized (Sangon Biotech, Shanghai, China). The total cellular RNA was extracted with mirVana™ miRNA Isolation kit (Ambion, USA) from the transgenic fibroblasts and the cells transfected with pEGFP-N1 and no plasmid. The total RNA was reversely transcribed into cDNA using reverse transcription kit (Fermentas, USA). The cDNAs were detected with PCR amplification. PCR program was as followings: 95°C for 10 min; 40 cycles of 95°C for 30 sec and 61°C for 30 sec and 72°C for 1 min; 72°C for 5 min. The products were detected with electrophoresis of 2% agarose gels.

RESULTS AND DISCUSSION

PCR amplification and T vector cloning of three genes: To clone the DNAs of three genes, researchers aligned

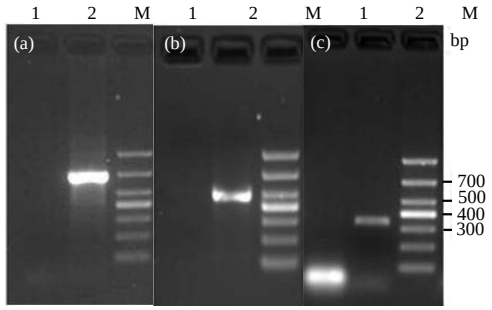

Fig. 1: PCR amplification products of three target genes. a-c) 1: PCR negative control; 2: GH, IGF-I and SST cDNA fragments, respectively. M: DNA marker

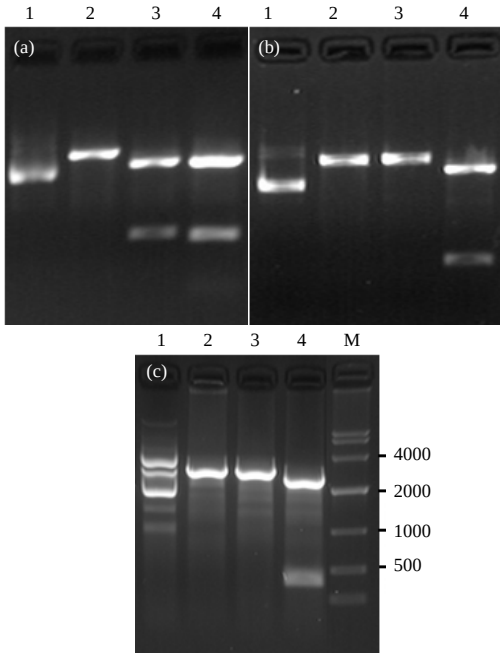

Fig. 2: Restriction endonuclease digestion of the three T vectors. a) pGEM-T-GH digestion; b) pMD18-T-IGF-I digestion. 1: plasmid; 2: plasmid plus BamHI; 3: plasmid plus SalI; 4: plasmid plus BamHI and SalI. c) pUCm-T-SST digestion. 1: plasmid; 2: plasmid plus XhoI; 3: plasmid plus KpnI; 4: plasmid plus XhoI and KpnI. M: DNA marker

earlier reported sequences of these genes of other species and found that the 5' and 3' ends of three genes were very conserved. Therefore, researchers designed three pairs of primers according to this kind of homology for preparing full-length cDNA of these genes using PCR amplification. The PCR primers for amplifying *GH* and *IGF-I* gene were designed based on the sequences of the Capra hircus (GenBank: Y00767.1 and FJ716104.1) and the

PCR primers for amplifying *SST* gene were designed based on the sequence of *Ovis aries* (GenBank: AF031488.1) using the software Prime Premier 5.0. The DNAs of three genes were amplified with these primers from cDNA reversely transcribed from total RNA isolated from the pituitary, liver and pancreas tissues of Anhui white goat. As a result, the DNA fragments of approximately 700, 500 and 350 bp were amplified for *GH*, *IGF-I* and *SST* genes, respectively (Fig. 1). As expected, the size of these fragments was in agreement with the length of coding region of these genes of other species.

Researchers next cloned three DNA fragments into T vector and constructed three plasmids: pGEM-T-GH, pMD18-T-IGF-I and pUCm-T-SST. To confirm the cloned sequences of three genes, researchers performed double-enzyme digestions to find whether the released DNA fragments were identical to PCR product in length.

The results revealed that the former was almost identical to the latter (Fig. 2). To further confirm the sequences of three genes, researchers sequenced the inserted DNAs of three plasmids. The results demonstrated that the sequences were in high homology with the known sequences coding these genes (Fig. 3). The full-length of GH, IGF-I and SST cDNAs obtained in this study were stored in GenBank and the accession number is JX570671, JX570672 and JX570673, respectively. The stop codons of *GH* and *IGF-I* genes were mutated (GH: TAG-TAT; IGF-I: TAA-TAT) to meet the requirements of the pEGFP-N1 vector with retaining their termination codon unchanged in sequence alignment.

Researchers compared cDNA and amino acid sequences of *GH* gene of Anhui white goat with those of Tokara goat (GenBank: X07035.1), Sarda breed goat (GenBank: GU355687.1), Beetal goat (GenBank:

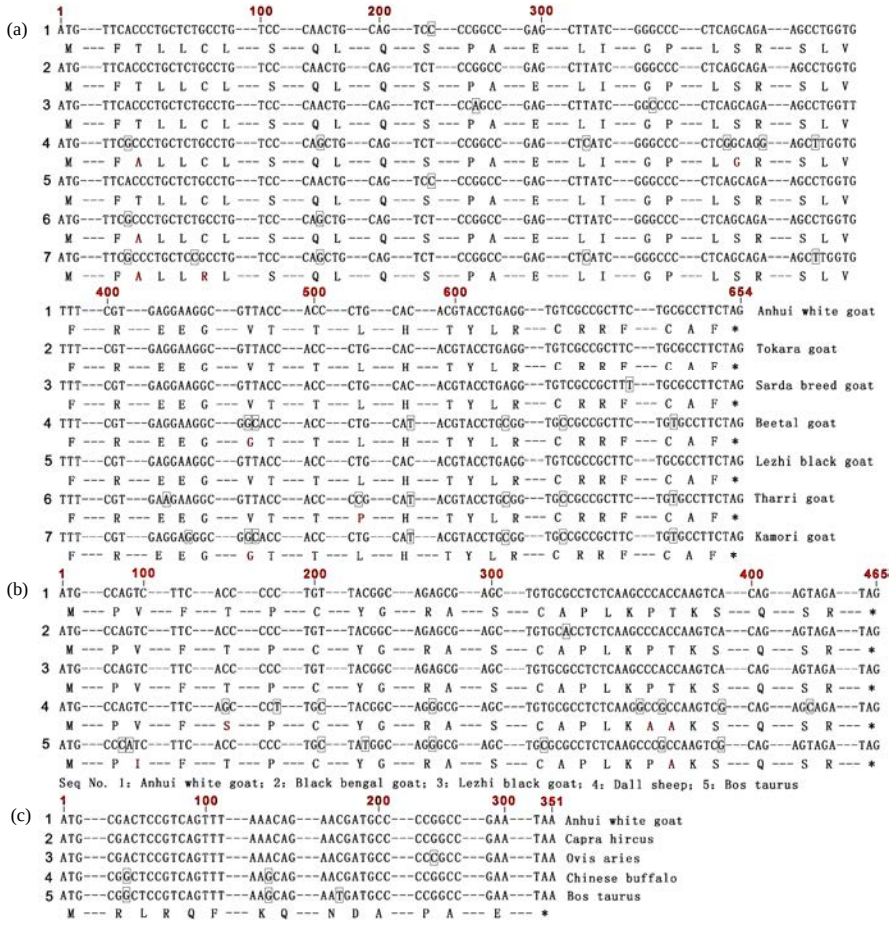

Fig. 3a-c: Alignment of cDNA and amino acid sequences of *GH*, *IGF-I* and *SST* genes of Anhui white goat and several other animals. Alignment of *GH*, *IGF-I* and *SST* genes, respectively. The bases within the frames are different from the ones of Anhui white goat; the dashed lines represent the same sequences. The amino acid sequences of several mammalian *SST* genes are identical, shown by single line of amino acid sequence

Table 1: cDNA and amino acid differences in the three target genes between species (or breed) and homology

Genes	Species/breeds	Base mutations	Amino acid changes	Homology
GH	Tokara goat	242 (T→C)	No mutation	99.85%
	Sarda breed goat	242 (T→C); 254 (A→G); 341 (C→G); 383 (T→G); 629 (T→C)	No mutation	99.24%
	Beetal goat	146 (G→A); 242 (T→C); 323 (C→T); 357 (G→A); 362 (G→A); 378 (T→C); 466 (G→T); 467 (C→T); 587 (T→C); 606 (C→A); 620 (C→T); 644 (T→C)	15 (Ala→Thr) 120 (Gly→Ser) 156 (Gly→Val)	98.01%
	Lezhi black goat	No mutation	No mutation	100%
	Tharri goat	42 (G→A); 146 (G→A); 242 (T→C); 428 (A→G); 541 (C→T) 587 (T→C); 606 (C→A); 620 (C→T); 644 (T→C)	15 (Ala→Thr) 181 (Pro→Leu)	98.62%
	Kamori goat	42 (G→A); 51 (C→T); 146 (G→A); 242 (T→C); 323 (C→T) 378 (T→C); 431 (G→A); 466 (G→T); 467 (C→T); 587 (T→C) 606 (C→A); 620 (C→T); 644 (T→C)	15 (Ala→Thr) 18 (Arg→Cys) 156 (Gly→Val)	98.01%
IGF-I	Black bengal goat	332 (A→G)	No mutation	99.78%
	Lezhi black goat	No mutation	No mutation	100%
	Dall sheep	130 (G→C); 152 (T→C); 200 (C→T); 257 (G→A); 342 (G→C) 345 (G→A); 353 (G→A); 428 (C→T)	44 (Ser→Thr) 115 (Ala→Pro) 116 (Ala→Thr)	98.28%
	Bos taurus	77 (C→A); 78 (A→G); 200 (C→T); 239 (T→C); 257 (G→A) 329 (C→T); 345 (G→A); 353 (G→A)	27 (Ile→Val) 116 (Ala→Thr)	98.28%
SST	*Capra hircus*	No mutation	No mutation	100%
	Ovis aries	281 (C→G)	No mutation	99.72%
	Chinese buffalo	89 (G→A); 134 (G→A)	No mutation	99.43%
	Bos taurus	89 (G→A); 134 (G→A); 194 (T→C)	No mutation	99.16%

DQ307368.1), Lezhi black goat (GenBank: JF896274.1), Tharri goat (GenBank: EF451797.1) and Kamori goat (GenBank: EU086738.1) using Clustal W Software. The results revealed that the homology was >98% (Table 1). Researchers also compared cDNA and amino acid sequences of *IGF-I* gene of Anhui white goat with those of Black bengal goat (GenBank: FJ716104.1), Lezhi black goat (GenBank: JF896275.1), Dall sheep (GenBank: HQ285865.1) and *Bos taurus* (GenBank: HQ324241.1). The homology was >98% (Table 1). The comparison of cDNA and amino acid sequences of *SST* gene of Anhui white goat with those of Capra hircus (GenBank: GU014693.1), *Ovis aries* (GenBank: Y15267.1), Chinese buffalo (GenBank: GQ864014.1) and *Bos taurus* (GenBank: DQ156121.1) revealed that the homology was up to 99% (Table 1). These results demonstrated that the sequence of functional genes is very conserved in species evolution.

Construction of eukaryotic expression vectors of three genes: For preparing the transgenic materials for goat breeding, researchers next constructed the eukaryotic expression vectors for three genes by transferring the inserted DNA fragments from T vectors into a eukaryotic expression vector, pEGFP-N1. The obtained eukaryotic expression vectors for three genes, *pEGFP-N1-GH*, *pEGFP-N1-IGF-I* and *pEGFP-N1-SST* were further confirmed by double-enzyme digestions (Fig. 4) and DNA sequencing. The results revealed that the eukaryotic expression vectors for three genes were

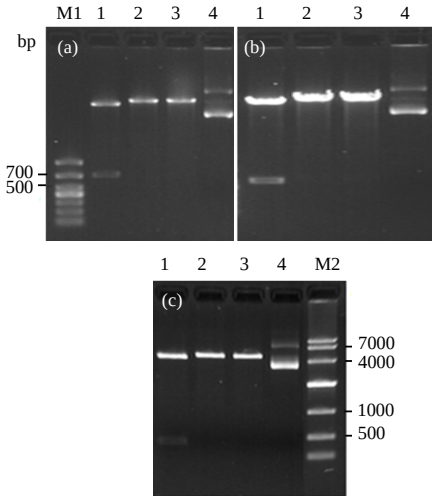

Fig. 4: Restriction endonuclease digestion of the three eukaryotic expression vectors. a) pEGFP-N1-GH digestion; b) pEGFP-N1-IGF-I digestion. 1: plasmid plus BamHI and SalI; 2: plasmid plus BamHI; 3: plasmid plus SalI; 4: plasmid; c) pEGFP-N1-SST digestion. 1: plasmid plus KpnI and XhoI; 2: plasmid plus KpnI; 3: plasmid plus XhoI; 4: plasmid. M1, M2: DNA markers

successfully constructed and the sequences of the inserted DNA fragments were identical to those in T vectors.

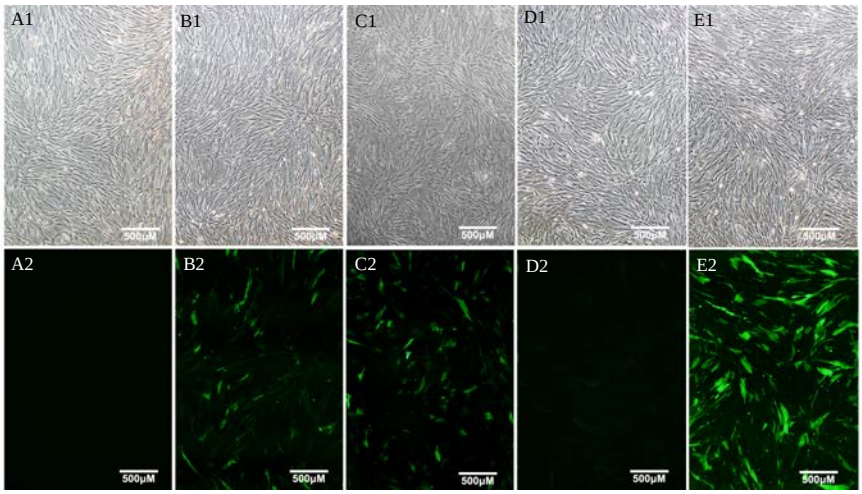

Fig. 5: The transient expression of transferred target genes in the transgenic fibroblasts of Anhui white goat. A) blank control cells; B-E) cells transfected with pEGFP-N1-GH, pEGFP-N1-IGF-I, pEGFP-N1-SST and pEGFP-N1, respectively. 1: bright field; 2: fluorescence. Scale: 500 μm

Manufacture and detection of stable transfection fibroblasts: To provide more direct transgenic materials for goat breeding, researchers subsequently transfected the fetal fibroblasts of Anhui white goat and selected the stable transfection clones of three genes. As a result, the transient expression cells (Fig. 5) and stable transfection clones (Fig. 6) of three genes were obtained. To verify the integration of target genes into the genome of fibroblast of Anhui white goat, the stable transfection clones were detected with a pair of pEGFP-N1 vector-specific PCR primers that flanked the inserted DNA fragment of target gene. As expected, the target genes were successfully amplified from the stable transfection clones (Fig. 7), revealing that the integrated exogenous target genes transcribed in the transgenic fibroblasts of Anhui white goat.

GH is a direct regulator of animal growth, therefore, *GH* gene were extensively used in the transgenic breeding of many agriculture-related species (Li *et al*., 2004). The IGF-I is thought to be the true growth regulatory factor of the livestock. It was reported that the growth promotion effect of GH is mediated by IGF-I. Therefore, in this study, researchers firstly cloned *GH* and *IGF-I* genes and prepared the stable transfection fibroblasts for transgenic breeding of Anhui white goat. In this study, researchers also performed similar studies to another growth-related gene, Somatostatin (*SST*) for exploring its potential application in transgenic breeding of Anhui white goat.

Somatostatin, a peptide hormone which is widely distributed *in vivo*, has a wide range of biological effects. Animal growth is mainly regulated by GH while the secretion of GH is under the control of Growth

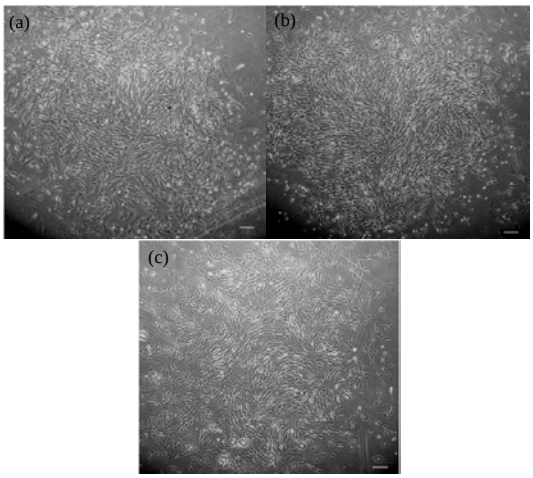

Fig. 6a-c: Positive clone cells. Positive clone cells transferred *GH*, *IGF-I* and *SST* genes, respectively. Scale: 200 μm

hormone-Releasing Hormone (GRH) and Somatostatin (SST) which are secreted from the hypothalamus, the former promoting GH secretion, the latter inhibiting GH secretion (Forsyth and Wallis, 2002; Lv *et al*., 2011). The SST expression was reduced in animals by inhibiting the *pre-SST* gene expression and the concentration of GH was increased correspondingly, as a result, the growth of animal was promoted (Wu *et al*., 2008). Therefore, SST itself can't be directly used to promote growth.

It was found that the immune of SST could improve the basic concentration of GH in sheep plasma (Varner *et al*., 1980). Therefore, the inhibition of SST can

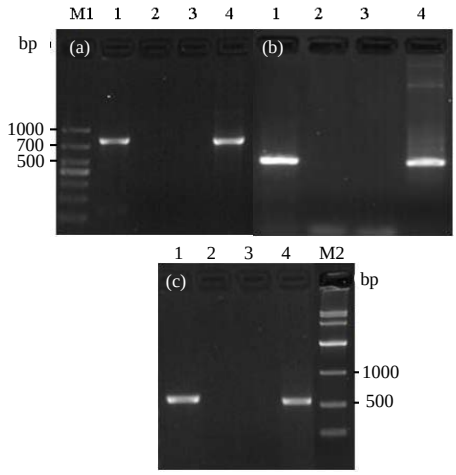

Fig. 7a-c: Expression of transferred genes in positive clone cells detected with RT-PCR. 1: cells transfected with pEGFP-N1-GH, pEGFP-N1-IGF-I and pEGFP-N1-SST, respectively; 2: cells transfected with pEGFP-N1; 3: blank control cells; 4: PCR amplification with pEGFP-N1-GH, pEGFP-N1-IGF-I and pEGFP-N1-SST plasmids as template, respectively. M1, M2: DNA markers

enhance the release of GH that can correspondingly promote growth. In this study, researchers cloned *SST* gene of Anhui white goat and reversely inserted it into the expression vector pEGFP-N1. In this way, researchers constructed a eukaryotic expression vector that can transcribe the antisense stand of *SST* gene. Researchers expected that the transcribed antisense strand can anneal to the indigenous mRNA of *SST* gene and thus reduce the expression level of SST hormone, correspondingly, increase the secretion of indigenous GH for growth promotion. An advantage of transferring reversed *SST* gene is that only its transcription is needed for growth promotion but as to GH and IGF-I, both transcription and translation are demanded for their growth promotion effect.

CONCLUSION

This study cloned three genes, *GH*, *IGF-I* and *SST*, from the pituitary, liver and pancreas tissues of Anhui white goat, constructed the eukaryotic expression vectors for these genes and screened the stable transfection fibroblasts of Anhui white goat. The PCR detection revealed that the transferred genes were integrated in genome of the transgenic fibroblasts and expressed in the transgenic fibroblasts. The obtained transgenic fibroblasts provide mature breeding materials for the transgenic breeding of this goat. Of course, the true growth promotion effects of these breeding materials on the live body should be further verified in the later *in vivo* studies.

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