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RESEARCH ARTICLE

Matrix attachment regions included in a bicistronic vector enhances and stabilizes follistatin gene expressions in both transgenic cells and transgenic mice

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Abstract In the present study, follistatin (*FST*) gene expression vectors with either a bicistronic gene transfer cassette alone, or a bicistron gene cassette carrying a matrix attachment region (MAR) were constructed and transfected to bovine fetal fibroblasts. Evaluations of both the integration and expression of exogenous *FST* indicated that the pMAR-CAG-FST-IRES-AcGFP1-polyA-MAR (pMAR-FST) vector had higher capacity to form monoclonal transgenic cells than the vector without MAR, though transient transfection and integration efficiency were similar with either construct. Remarkably, protein expression in transgenic cells with the pMAR-FST vector was significantly higher than that from the bicistronic vector. Exogenous *FST* was expressed in all of the pMAR-FST transgenic mice at F_0, F_1 and F_2. Total muscle growth in F_0 mice was significantly greater than in wild-type mice, with larger muscles in fore and hind limbs of transgenic mice. pMAR-FST transgenic mice were also found with more evenly distributed muscle bundles and thinner spaces between sarcolemma, which suggests a correlation between transgene expression-associated muscle development and the trend of muscle growth. In conclusion, a pMAR-FST vector, which excluded the resistant genes and frame structure, enhances and stabilizes *FST* gene expressions in both transfected cells and transgenic mice.

Keywords safety of transgenic, bicistron gene transfer body, transgenic mice, muscle development

1 Introduction

Safety issues arising from genetically modified organisms (GMO) are of increasing concern, especially for commercialization of transgenic plants and animals. Generally, recombinant plasmids composed of a backbone sequence, plus resistance and marker genes are used for transgenic purposes. Random integration and inheritance of these elements may generate potential risk factors for the resultant GMO[1–3]. Muller et al. demonstrated that a vector backbone sequence containing a palindrome structure could induce stable secondary structure between plasmids and resulted in illegitimate recombination[2]. Philip et al. reported that RNAs transcribed from vector backbone sequences could potentially interfere with the synthesis and processing of RNA from targeted genes. It is likely that vector backbone sequences could cause the occurrence of multiple copies of transgenes[4]. Stoger et al. indicated that co-integration of both exogenous vector sequence and the targeted gene sequence could result in formation of giant transgenic loci, which could potentially induce formation of an unstable three-dimensional structure leading to the loss of the locus and the silencing of target genes. Currently, Cre/Loxp, FLP/FRT or R-RS systems are used to delete unnecessary and useless genes[5–7]. Although the Cre/Loxp recombination enzyme system can remove resistance or marker genes, this still needs a secondary redundant transfection[7]. Using similar recombinant plasmids, plants have been found with linear DNA with transformation, integration and genetic stability, which only contain a promoter and an open reading frame and a terminator[3,4,8]. However, linear DNA vectors without a backbone sequence, and selectable and resistant genes have not been used in transgenic animals.

A matrix attachment region (MAR) is a specific type of DNA sequence that widely exist in the boundary sequence of the chromatin loop structure, which may combine with

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the nuclear matrix *in vitro*[7,9,10]. Analysis of the sequence of MARs indicated that all of the known MARs have common features, including A + T richness (> 70%) and sequence motifs, such as the A-box (AATAAAT/CAAA), T-box (TTATTT/ATTT/ATT), autonomously replicating sequence in yeast (ARS), recognition site of *Drosophila* topoisomerase II, and unwinding DNA for recognition of proteins and curved DNA[9–11]. In plants, MARs are not only important in chromatin folding and regulation of expression of adjacent endogenous genes, but also influence exogenous gene expression, when added on both sides of these genes[9,10]. In animals, using expression vectors containing MAR elements could avoid the occurrence of silenced cells, as well as clonal heterogeneity[11]. Several MARs are known to enhance protein production in transgenic cells containing human MARs at β-globin and IFN-loci, or the chicken lysozyme locus[11–13]. Therefore, it is important to investigate whether activation of MARs can improve transgene expression in transgenic animals when used as transgene regulators.

Based on previous findings of the positive influence of MAR sequences on integration and expression of exogenous gene in transgenic mice, MAR sequences derived from cattle are hypothesized to have a similar function. In the present study, a vector with a bicistronic gene cassette with MARs added at both ends, but without a bacterial sequence and resistant gene, was constructed as pMAR-CAG-FST-IRES-AcGFP1-polyA-MAR (or pMAR-FST). Its efficacy for transfection, integration and expression were first analyzed in transgenic cells. This pMAR-FST vector was then used to generate transgenic mice. pMAR-FST mice had skeletal muscles that were significantly enlarged when compared to control wild-type mice, suggesting that MAR sequence with *FST* bicistronic gene-transfer cassette was more appropriate for transgenic applications. To our knowledge, this is the first successful application of bovine MAR sequence along with *FST* and a bicistronic transfer cassette in transgenic animals.

2 Materials and methods

2.1 Ethics statement

Experimental procedures were approved by the Animal Care Commission of the College of Biological Sciences, Inner Mongolia University.

2.2 Chemicals

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Disposable, sterile plastic wares were purchased from Nunclon (Roskilde, Denmark).

2.3 Construction of vectors

The correctly sequenced *FST* gene was first inserted into vector pCAG-IRES-AcGFP1-polyA to construct the vector pCAG-FST-IRES-AcGFP1-polyA. Then two correctly sequenced MAR genes were attached to the vector pCAG-FST-IRES-AcGFP1-polyA, one before the CAG promoter and the other after the polyA signal. This resulted in the vector pMAR-CAG-FST-IRES-AcGFP1-polyA-MAR. The bicistronic gene cassette, pCAG-FST-IRES-AcGFP1-polyA, was produced by digestion of the plasmid pCAG-FST-IRES-AcGFP1-polyA with restriction enzymes *Sac*I and *Afl*II followed by separation and extraction using agarose gel electrophoresis. The bicstronic gene cassette with MAR at both ends (pMAR-CAG-FST-IRES-AcGFP1-polyA-MAR) was produced from the plasmid pMAR-CAG-FST-IRES2-AcGFP1-polyA-MAR by the same procedure (Fig. 1, Appendix A).

2.4 Culture and transfection of bovine fetal fibroblast cells

Bovine fetal fibroblasts were isolated from a 50- to 60-day-old cattle fetus by disaggregation of the body without head and viscera, followed by culturing in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco) at 38.5°C in a humidified atmosphere with 5% CO_2. At confluence, the cells were collected by trypsinization for passaging or cryopreservation. The cells were seeded into 12-well plates and cultured in fresh DMEM without antibiotics to achieve 80% to 90% of confluence on the day of transfection. The cells were then transfected with Lipofectamine™ LTX according to the manufacturer's instructions. Briefly, for each well of cationic lipid

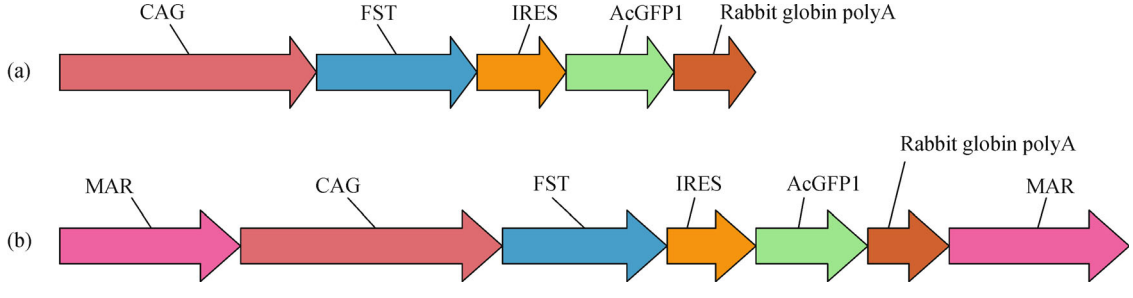

Fig. 1 Construction maps of vectors. (a) Bicistronic gene cassette; (b) pMAR-FST.

transfection, 800 ng of plasmid DNA (1 μg·μL^{-1}) was added to 100 μL Opti-MEM, mixed thoroughly and then 2 μL PLUS reagent added directly to the diluted DNA, mixed gently before incubating at room temperature for 5 min. LTX reagent (3.5 μL) was added to 100 μL Opti-MEM, and then added to the DNA solution, before mixing thoroughly. This mixture was incubated for 30 min at room temperature. The DNA-lipid complexes were added dropwise to the wells containing cells and gently mixed by rocking the plate back and forth. The cells were incubated at 37°C in a CO_2 incubator for 6 h, and then changed growth medium.

After culture for 48 h, single cells were sorted to 96-well plates by a flow cytometer for continued culture. The single cells that expressed AcGFP1 were identified as transfectants. After culture for 7 days, single cells were sorted in the same way. The single cells that stably expressed AcGFP1 indicated the exogenous had been integrated into the recipients' chromosome. These single cells were then cultured to form mono-clones. The fluorescence and proportion of positive cells were observed and measured by an inverted fluorescent microscope and flow cytometry, respectively. The negative control group was included to eliminate any error resulting from the instrument.

2.5 Quantitative real-time PCR

Total RNA was extracted from each sample using Trizol reagent (Invitrogen, New York, USA), and reverse transcription was performed to generate cDNA using a PrimeScript™ RT reagent kit (TaKaRa, Dalian, China). The samples were analyzed with SYBR Premix Ex Taq™ II (TaKaRa). For each sample, target and reference genes were amplified independently on the same plate and in the same experimental run in triplicate. PCR specificity was confirmed by gel electrophoresis on a 2.5% agarose gel and by the presence of a single peak in the melting curve. For relative quantitative RT-PCR, the amount of target normalized to the reference was calculated by the $2^{-\Delta\Delta C_T}$ method.

2.6 Western blot analysis

The cells were harvested 48 h after transfection, rinsed with phosphate-buffered saline and lysed in 150 μL of ice-cold RIPA buffer composed of 50 mmol·L^{-1} Tris, 150 mmol·L^{-1} NaCl, 0.5% Na deoxycholate, 1% Nonidet P-40 and 0.1% sodium dodecyl sulfate (SDS). Then, the cell lysates were centrifuged at 4°C for 5 min at 8000 *g*. The supernatant was electrophoresed in an 8% SDS-polyacrylamide gel and then transferred onto the polyvinylidene difluoride membrane by performing electroblotting. The membrane was blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) blocking solution at room temperature for 1 h and subsequently incubated with FLAG-specific monoclonal antibody diluted 1:1000 (F1084, Sigma, USA) in TBST. The membrane was then incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse secondary antibody at 1:1000 in TBST, followed by detection using the chemiluminescence labeling detection reagent ECL Plus (Thermo Scientific, Shanghai, China).

2.7 Monoclonal cells selection and identification

The sorted single transfected cells were infinitely diluted into 100 mm dish, to keep cells far apart from each other to guarantee they grew into monoclones, and cultured for 6–9 days. The mono-clones were selected and passaged in 24-well plates. After several passages, the stable cell lines were identified.

The genomic DNA of each cell line was extracted by genome extraction kit and the integration of exogenous genes was detected by PCR. Two pairs of primers were designed to detect the integration of bicstronic of FST-AcGFP1 and MAR-FST. The primers (5′–3′) were: FST-AcGFP1, JCF1 sense AACTTCGGGCTTTGCCTCC TGCT, JCR2 and antisense AACTTGCTCATCCAT GCCGTGGG; MAR-FST, JCF1 sense AACTTCGGGC TTTGCCTCCTGCT; and CMAR2 antisense AATCGG TCGACTGAGTCATCCTTTCCTTG.

Due to *FST* being an endogenous gene expressed in normal bovine fetal fibroblasts, only the co-expression of bicstronic FST-AcGFP1 was detected. Total RNA of each cell line was extracted and tested for the expression of exogenous genes by RT-PCR.

2.8 Transgenic mice preparation, breeding and identification

To test if the pMAR-FST vector functions normally at the physiological level, transgenic mice (F_0) were prepared by pronuclear injection using the pMAR-FST vector. After hybridization of F_0 founders with wild-type mice F_1 and F_2 mice were obtained. At 4-week-old, the tails of the F_1 and F_2 mice were cut to extract genomic DNA, and PCR was performed to determine whether the mice were transgenic. The primers (5′–3′) were GF-F sense GCTGGTTATTGTG CTGTCTC and GF-R antisense TCCTGGTCTTCAT CTTCCTC. The total RNA of muscle tissues was extracted from both transgenic and wild-type mice, and reverse transcribed into cDNA to detect the expression of muscle development related genes and the exogenous genes by real-time PCR. Designed primers *MSTN*, *MYoD*, *MYoG*, *PAX3*, *GAPDH*, *AcGFP* and *FST* are given in Table 1.

2.9 Animal care and muscle histological analysis

All experimental mice were maintained under the following conditions: temperature of 25±1°C with 70%±4% relative humidity, automatic light control, rearing density

Table 1 Primers for real-time PCR analysis

Gene name	Primers	Sequences (5′–3′)
MSTN	Forward	GCTCAAACAGCCTGAATCCAACTTA
	Reverse	CGCAGTCAAGCCCAAAGTCTC
MYoD	Forward	TGACCCGTGTTTCGACTCC
	Reverse	GCAGGGAAGTGCGAGTGTT
MYoG	Forward	CGAGTGCCCCTTGAAGACA
	Reverse	CCGACTTCCTCTTACACACCTTACA
PAX3	Forward	GTCCCATGGCTGCGTCTCTAA
	Reverse	TCTCCACGTCAGGCGTTGTC
GAPDH	Forward	AAATGGTGAAGGTCGGTGTGAAC
	Reverse	CAACAATCTCCACTTTGCCACTG
AcGFP	Forward	ATGGCAACATCCTGGGCAATAAGAT
	Reverse	CGCCGATGGGGGTATTCTGCTGGTA
FST	Forward	GAAAAACCTACCGCAACGAATGTG
	Reverse	ATTATTAGTCTGGTCCACCACGCA

less than or equal to four per cage. Mice were provided food and water ad libitum. All mice were anesthetized before being euthanized.

Small sections of muscle tissue were taken from the forelimb and hindlimb of both F_0 transgenic and wild-type mice. The tissues were fixed in 4% paraformaldehyde, and embedded in paraffin. Five-micrometer-thick sections were deparaffinized and stained with hematoxylin-eosin and pictures were taken from four random fields at 400 × magnification under a microscope (DS-Ri1, Nikon) (http://imagej.net) was used to calculate the muscle cross-sectional area.

2.10 Statistical analysis

Each experiment was performed at least three times. All data were analyzed using SPSS 20.0 statistical software (IBM Corporation, Somers, NY, USA). Data were tested by one-way ANOVA and least-significant difference tests, and reported as the mean and SEM. For all analyses, $P < 0.05$ was considered significant.

3 Results

3.1 Transfection and integration efficiency

Results from assays of both fluorescence microscopy and flow cytometry indicated that there was no significant difference in the transient transfection efficiency between the bicistronic gene cassette (17.4%) and pMAR-FST (21.0%) (Table 2). In addition, there was also no significant difference in integration rate between the bicistronic gene cassette (0.6%) and pMAR-FST (1.3%) (Table 3).

3.2 Comparison of *FST* RNA expressions between transgenic cells with bicistronic or pMAR-FST gene cassette

cDNAs from *FST* mRNA were generated from transgenic cells with the bicistronic cassette, pMAR-FST and negative control, in order to detect the *FST* mRNA expression by Real Time PCR; β-actin was as the internal control. As shown in Fig. 2, setting the negative control group to 1, the level of *FST* expression in pMAR-FST was 19.4,

Table 2 Comparison of transfection efficiency

Experimental repeats	1	2	3	Mean
Bicistronic gene cassette/%	19.31	11.83	21.03	17.39±3.99
pMAR-FST/%	23.16	14.07	25.93	21.05±5.06
Negative control/%	0.15	0.24	0.12	0.17

Table 3 Comparison of the integration efficiency

Experimental repeats	1	2	3	Mean
Bicistronic gene cassette/%	0.85	0.39	0.59	0.61±0.19
pMAR-FST/%	1.36	1.48	1.18	1.34±0.12
Negative control/%	0.04	0.06	0.06	0.05

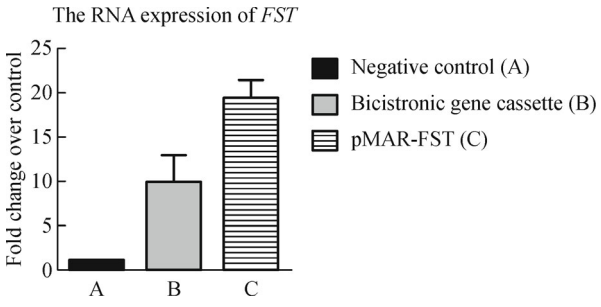

Fig. 2 *FST* mRNA expression by real-time PCR

significantly higher than the 7.35 obtained with the bicistronic cassette.

Together, these results indicated that pMAR-FST enhanced *FST* expression compared to the bicistronic construct.

3.3 Comparison of FST protein expressions in transgenic cells with either the bicistronic gene or pMAR-FST constructs

The FST expression with the bicistronic gene construct was similar to the negative control. However, the pMAR-FST transfected cells had significantly higher expression of exogenous FST when compared to the bicistronic gene construct transfected cells or the controls. No significant difference in GAPDH expression was observed between the different groups. Together, these results suggested that the MAR sequence may increase the expression of exogenous *FST* genes. The mechanism, however, needs further study (Fig. 3).

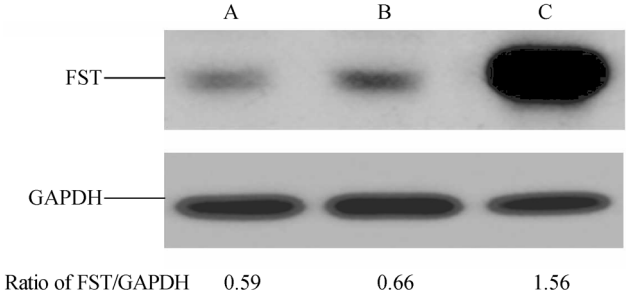

Fig. 3 FST translation in bovine fetalblasts. FST protein after translation was detected by western blot assay. The mean grave value was measured by using Imagej software. A, The negative control; B, bicistronic gene cassette; C, pMAR-FST vector.

3.4 Selection and characterization of monoclonal transgenic cells

3.4.1 Selection of monoclonal cells

To build a secure gene transfer cassette that did not contain resistance genes or other additional frame sequence, we analyzed the transgenic cells by standard antibiotics screening methods after transfection. In the pMAR-FST group, positive clones formed in 5 days after confection, while the monocloned cells were observed until 9 days after confection with the linear vector. A total of 23 monoclones in the pMAR-FST group were obtained and eight of them were transgenic. Four monoclones were obtained from the group containing the bicistronic gene construct and one of them was transgenic. The clones from the pMAR-FST vector were larger and grew more vigorously than those from clones with bicistronic gene cassette vector (Fig. 4).

3.4.2 Characterization of the integration of FST-AcGFP1 and FST-MAR sequences in transgenic cells

To determine whether the whole pMAR-FST vector was integrated into the genome, sequences from FST-AcGFP1 and FST-MAR were examined. Genomic DNA of eight transgenic monoclonal cells were extracted and tested by PCR. The results showed that FST-AcGFP1 had been integrated in all eight of the transgenic cell lines (Fig. 5) and that FST-MAR had been integrated in seven of the eight transgenic cell lines (Fig. 5), which indicated that seven of the eight transgenic monoclonal cells had the pMAR-FST fully integrated into genome of host cells.

3.4.3 mRNA expression in the transgenic cells

The mRNAs were isolated from eight pMAR-FST transgenic cells and then copied into cDNA. The level of FST mRNA expression was detected by real-time PCR, β-actin was used as the internal control. The results showed that FST-AcGFP1 was expressed in all eight transgenic cell lines (Fig. 6).

3.5 Characterization of transgenic mice

3.5.1 Breeding of transgenic mice

Three founder (F_0) pMAR-FST transgenic mice were obtained consisting of two males and one female. The F_0 male mice were mated with wild-type C57BL/6J female mice and four heterozygous F_1 and four F_2 transgenic mice were obtained (half siblings) (Fig. 7). The mRNAs were isolated from all of the F_0, F_1 and F_2 pMAR-FST transgenic mice and reverse transcribed into cDNA to detect mRNA expression of exogenous genes by real-time PCR. The results showed that the exogenous genes were expressed in all of the pMAR-FST transgenic mice (Fig. 8).

3.5.2 Expression of genes associated with muscle development

To analyze whether the expressions of muscle development

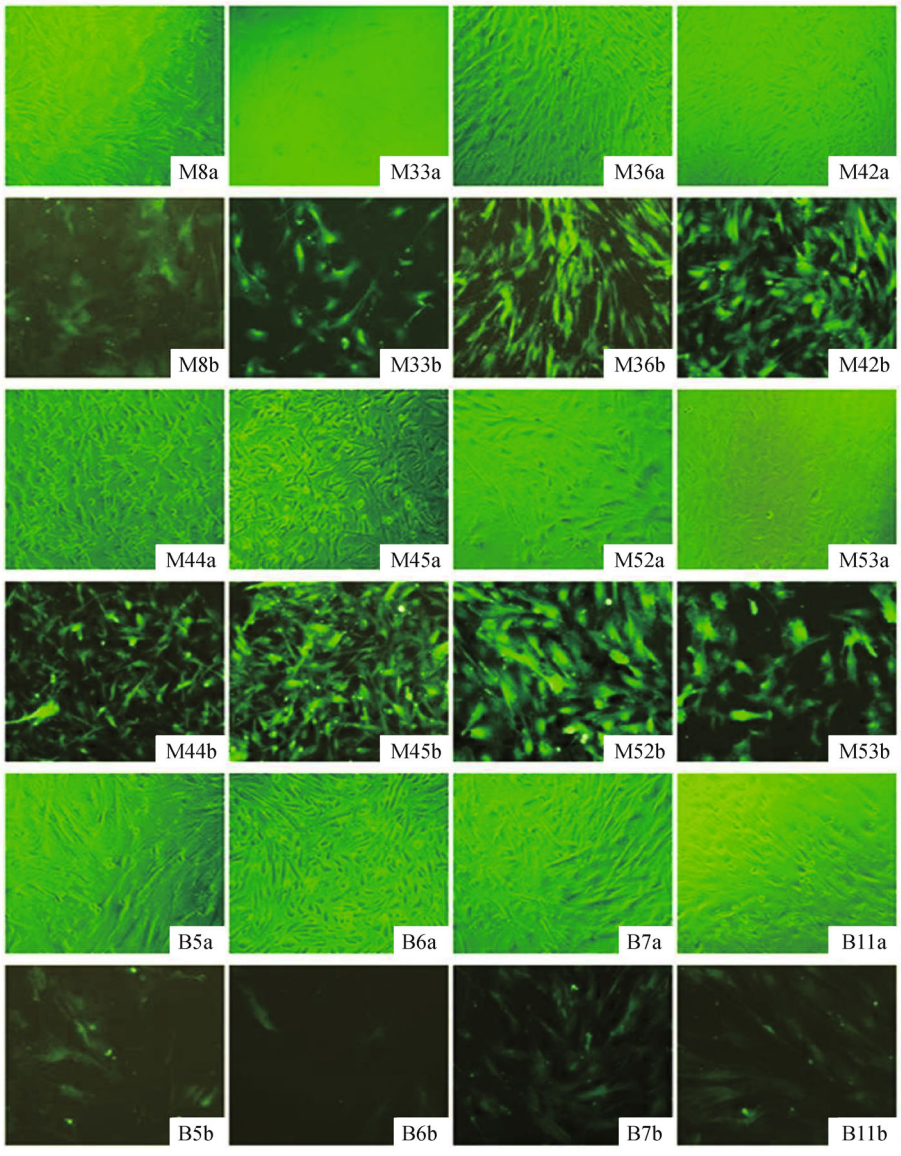

Fig. 4 Transgenic cell monoclones obtained from different vectors. a, Growth state of transgenic cells under the bright-field; b, fluorescence levels of transgenic cells under the dark field. M8, M33, M36, M42, M44–45, M52–53, Monoclone of transgenic cells with pMAR-FST vector; B5–7, B11, monoclone of transgenic cells with bicistronic gene cassette.

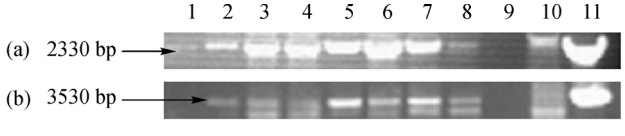

Fig. 5 PCR analysis of FST-AcGFP1 (a) and FST-MAR integration (b). Lanes assigned as: 1, M8; 2, M33; 3, M36; 4, M42; 5, M44; 6, M45; 7, M52; 8, M53; 9, control of ddH_2O; 10, negative control; 11, positive control.

related genes changed in F_0, F_1 and F_2 pMAR-FST transgenic mice, *MSTN* (myostatin), *MYoG*, *MYoD* and *PAX3* were each assayed by real-time PCR. The results

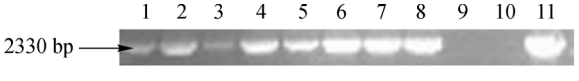

Fig. 6 RT-PCR analysis on RNA expressions.1, M8; 2, M33; 3, M36; 4, M42; 5, M44; 6, M45; 7, M52; 8, M53; 9, control of ddH20; 10, negative control; 11, positive control.

showed that *MSTN* was upregulated, while *MYoG*, *MYoD* and *PAX3* were downregulated in the F_0 pMAR-FST transgenic mice in comparison with the wild-type mice. The expression of *MSTN*, *MYoG*, *MYoD* and *PAX3* were all downregulated in F_1 and F_2 pMAR-FST transgenic mice (Fig. 9).

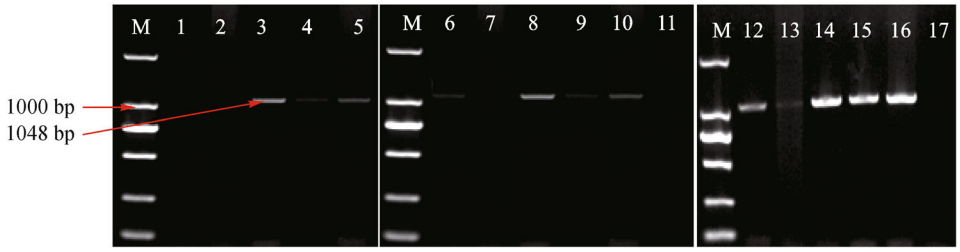

Fig. 7 PCR analysis on *FST* gene in transgenic mice. M, DL2000 Marker; 1, 2, 7, 11, 17, the control of ddH_2O; 3–5, the primary generation of transgenic mice; 6, 8–10, the first generation of transgenic mice; 12–15, the second generation of transgenic mice; 16, the plasmid of positive control.

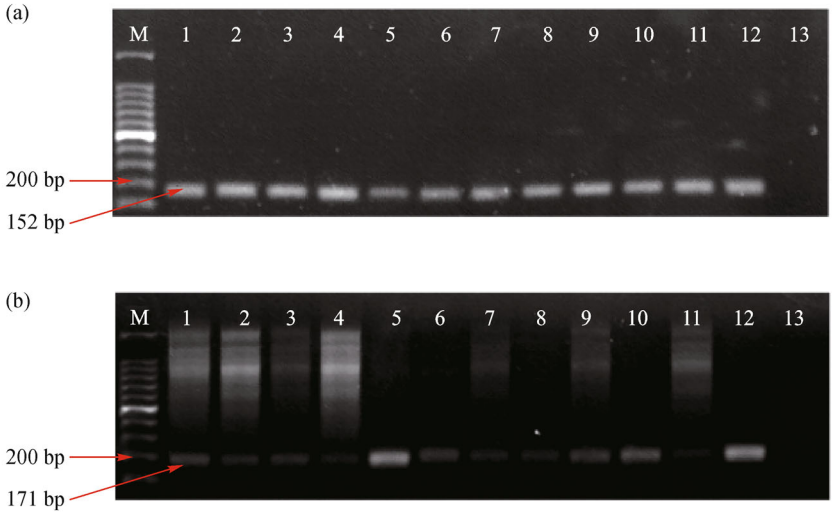

Fig. 8 RT-PCR analysis on exogenous genes expressions. (a) RT-PCR analysis of *FST* expression; (b) RT-PCR analysis of *AcGFP* expression. M, 100bp Marker; 1–3, primary generation of transgenic mice; 4–7, the first generation of transgenic mice; 8–11, the second generation of transgenic mice; 12, plasmid of positive control; 13, control of ddH_2O.

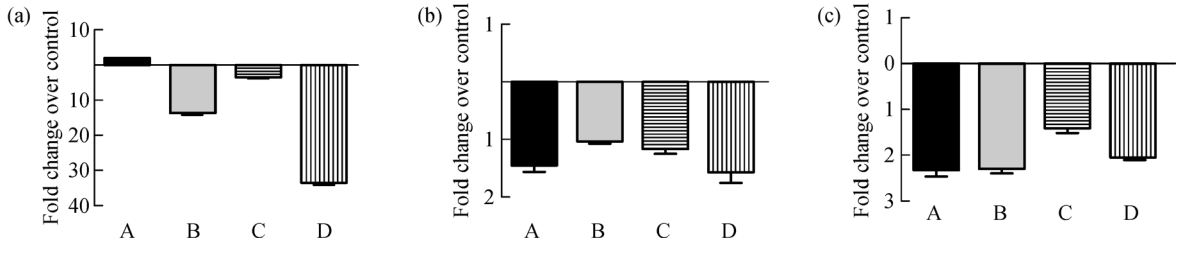

Fig. 9 Expressions of *FST* enhanced muscle development in transgenic mice. The fold changes of muscle development related gene between *FST* transgenic mice and wild-type mice were calculated from the results after qRT-PCR assays. (a) F_0 transgenic mice; (b) F_1 transgenic mice; (c) F_2 transgenic mice. A, *MSTN*; B, *MyoG*; C, *MyoD*; D, *PAX3*.

3.5.3 *FST* transgenic mice induced enhanced muscle development

Anatomical analysis showed that the whole body lengths of the F_0 pMAR-FST transgenic mice were similar to the wild-type mice, both being 110 mm (Fig. 10a). However, the fore and hind limbs of pMAR-FST transgenic mice were 24 and 30 mm, respectively, which were longer than the wild-type mice (17 and 23 mm, respectively). The bodyweight of pMAR-FST transgenic mice were 49 g, which was heavier than the wild-type mice at 42 g (Fig. 10b).

Histological analysis revealed that the muscle bundles were distributed more evenly, and the inter-space between myofibers in pMAR-FST transgenic mice (Fig. 10c) were thinner than that in wild-type mice (Fig. 10d). The average myofiber diameter was 4510 μm^2 in pMAR-FST transgenic mice, which significantly larger than wild-type mice with an average of 2740 μm^2.

4 Discussion

A MAR is a fragment of DNA sequence that can specifically combine with the nuclear matrix and anchor DNA or chromatin to it[10]. As natural parts of the eukaryotic genome, MAR sequences act as the boundary elements which isolate protect individual genes from the influence of some control elements in adjacent areas. Argyros et al. reported that development of S/MAR minicircles enhanced persistent expression of a transgene in mouse liver[14]. In the present study, bovine MAR sequence was isolated from the bovine genome and added to both sides of an *FST* bicistronic gene construct from which the resistant gene and bacterial frame structure had been deleted. The *FST* bicistronic gene construct with MAR was used for delivery into both mouse fetal fibroblasts and fertilized oocytes for generating transgenic mice. Similar transfection efficiencies were shown using both MAR-added and classic MAR-free vectors, which indicated that deletion of bacterial sequence did not affect transfection. Moreover, using pMAR-FST vector resulted in significantly higher expression levels of both mRNA and protein than using MAR-free vector as controls. Compared with the results with the control vector, the monoclonal cells with MAR-mediated vector formed more rapidly and were larger in size. Bicistronic gene constructs carrying MARs formed positive clones more rapidly, which may be because the MAR sequence can more effectively change the cell state and promote cell proliferation, so that the expression of transgenes tends to be stabilized. Previous studies have confirmed that MAR sequence can enhance the expression level of an exogenous gene and the expressions tends to be more stable[13]. These results suggest that the use of MAR sequences improves both exogenous gene integration and expressions of mRNA and protein, as well as the efficiency of formation of monoclonal cells.

FST is known to be a gene for upregulation of muscle growth, promoting muscle development through the prevention of MSTN binding to its receptor[15]. Previous studies have shown that mice with the *FST* homozygous mutation resulted in significantly decreased muscle development[15–17]. In the pMAR-FST transgenic mice, *FST* as exogenous gene was detectable in DNA, RNA and protein of all F_0, F_1 and F_2 mice, which demonstrated that the backbone sequences-free MAR-bicistronic-gene-transfer cassette could integrate into the genome and passes from generation to generation. Gene expression associated with muscle development was consistent with the tendency of muscle physiological growth with *FST* overexpression in all of F_0, F_1 and F_2 transgenic mice. *MSTN* expression was

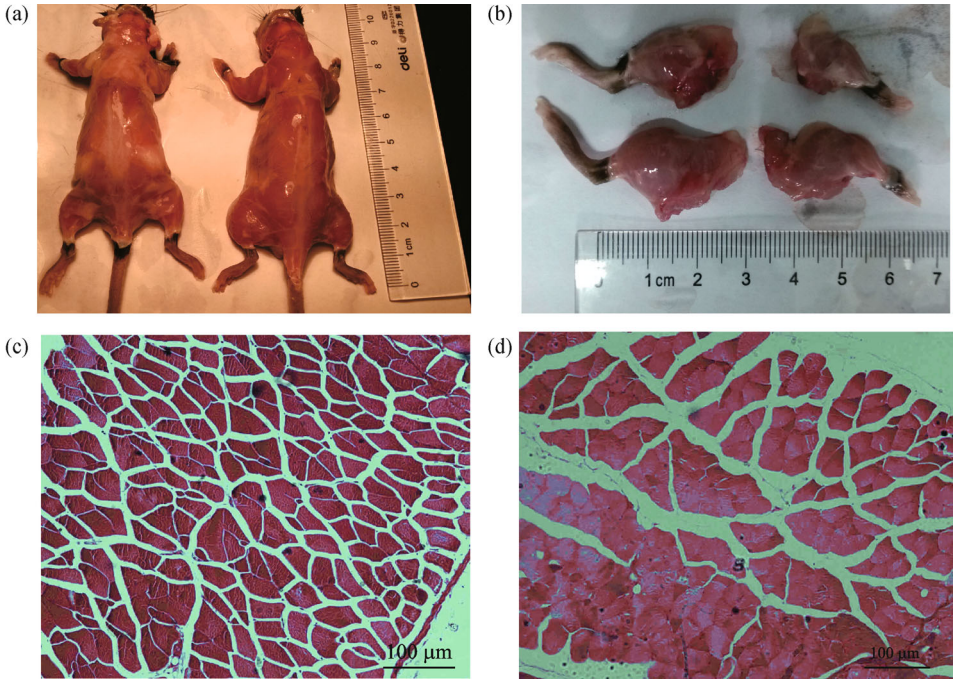

Fig. 10 *FST* transgenic mice induced strong muscle development with the increased muscle bundles. (a) The body length of transgenic mice (left) and wild-type mice (right); (b) the limbs of transgenic mice (left) and wild-type mice (right); (c) histological analysis on muscle tissues from primary generation of transgenic mice; (d) histological analysis on muscle tissues from primary generation of wild-type mice.

upregulated, while the expressions of *MYoG*, *MYoD* and *PAX3* were all downregulated in the F_0 transgenic mice. In addition, expression of *MSTN*, *MYoG*, *MYoD* and *PAX3* were all downregulated in both F_1 and F_2 transgenic mice. The upregulation of *MSTN* in F_0 transgenic mice probably reflected the compensation due to overexpression of *FST*[18]. The downregulation of *MSTN* in F_1 and F_2 transgenic mice probably shows that a functional balance of *FST* and *MSTN* can be established, and *MSTN* can be downregulated[18]. All the other genes examined, including *MYoG*, *MYoD* and *PAX3*, were also downregulated, suggesting the same effect of *FST* overexpression in all generations of transgenic mice.

In the present study, *FST* overexpression resulted in increased muscle mass growth in transgenic mice (Fig. 10). The limb weight and muscle mass in transgenic mice significantly increased in comparison to the wild-type mice. The results of histological analysis showed that the average myofiber diameters in *FST* overexpressing mice were significantly larger than that in wild-type mice (Fig. 10). These phenomena were most likely similar to the findings with *MSTN*-mutated mice[19,20]. This suggests that the *FST* gene carried by MAR sequence can be expressed effectively in the individual and play corres-ponding functions.

5 Conclusions

To our knowledge, this is the first study using MAR sequences to regulate *FST* for the delivery of exogenous genes in both transgenic cells and transgenic animals. Results indicated that the MAR with an *FST* bicistronic gene cassette not only stabilized integration, but also enhanced the expression of the exogenous *FST* gene in both transgenic cells and transgenic mice.

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Supplementary materials The online version of this article at http://dx.doi.org/10.15302/J-FASE-2016087 contains supplementary material (Appendix A).

Compliance with ethics guidelines Xiaoming Hu, Jing Guo, Chunling Bai, Zhuying Wei, Li Gao, Tingmao Hu, Shorgan Bou, and Guangpeng Li declare that they have no conflict of interest of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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