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Designing and Development of Anti-Myostatin Short Hairpin RNA (Shrna) Constructs for *Capra hircus*

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Abstract: The evolutionarily conserved process whereby small double-stranded (ds) RNAs sequence-specifically suppress the expression of their target genes is referred to as RNA interference (RNAi). Short hairpin RNA is about 55 nucleotide long RNA hairpin, consisting of a stem of 19-21 bp linked to a small, about 9 nucleotide terminal loop and bears a 2 nucleotide 3' overhang. Usually, the DNA sequence transcribing the shRNA is inserted into a vector containing the promoter. The expressed shRNA is then exported into the cytoplasm where it is processed by dicer into siRNA which then triggers RNA interference by activating RNA induced silencing complex (RISC). Myostatin is a major gene and its protein is a negative regulator of skeletal muscle growth. The present investigation was undertaken to design and clone the shRNA constructs against goat *(Capra hircus)* myostatin gene. The positive clones were screened by PCR and Restriction enzyme digestion. The sequencing of shRNA sequence was carried out using vector specific primers to detect any mutation (insertion or deletion) in its sequence. Four different constructs (sh1, sh2, sh3 and sh4) were developed in the present investigation. These constructs would be helpful in further experimental studies to investigate the *in-vitro* and *in-vivo* knockdown effect of MSTN gene in goat.

Key words: Short hairpin RNA · SIRNA · Myostatin · Sequencing · Cloning

INTRODUCTION

Short hairpin RNAs (shRNAs), encoded within the expression vectors have proven to be an effective mean of harnessing the RNA interference (RNAi) pathway. ShRNA is composed of a sense strand, connecting loop sequence and an antisense strand, which gives it the appearance of a hairpin structure. This design is based on the structure of naturally occurring miRNAs which are highly conserved noncoding RNA species that perform regulatory functions in organisms including sequence-specific repression of mRNA translation [1]. Like siRNA molecules, shRNAs can be produced synthetically although they are more commonly expressed by a plasmid or viral vector using a single promoter. Furthermore, reports have shown that shRNAs can also mediate mRNA degradation through processing of shRNAs into siRNAs by Dicer [2]. One of the first experiments involving shRNAs was silencing of firefly and luciferase reporter plasmids [2]. In this experiment, the suppression of gene expression by shRNAs was around 80-90%.

RNAi can be used to specifically knock down a gene in the cell which can be helpful in the identification of the components necessary for a particular cellular process. Myostatin (MSTN), a potent regulator of skeletal muscle is expressed predominantly in skeletal muscle. It acts as a negative regulator of skeletal muscle growth by suppressing proliferation and differentiation of myoblast [3, 4]. It was reported that the deleted myostatin gene in mice using a technique referred to as gene "knockout," generated mice that exhibited increased muscle mass and decreased fat tissue [3].

Knocking down of myostatin gene can have potential benefits for livestock producers as it can increase live weight, accelerate weight gain, increase carcass weight, improve feeding efficiency and reduce waste. For consumers, meat with increased protein, reduced fat and increased tenderness can be produced by utilizing this technique. Out of various species of meat consumed by Indian population, the goat meat is liked and acceptable by majority of the population. Knockdown of this gene in goat by developing a shRNA expressing construct

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against it will ultimately lead to the increased muscle production. Only a few reports are available about development of shRNA expressing construct against myostatin gene in any livestock species [5]. Perusal of literature revealed no report on the designing and development of shRNA expressing construct in any Indian goat breed. Therefore, the investigation was carried out to design and develop shRNA expressing constructs against goat MSTN gene.

MATERIALS AND METHODS

Designing of Anti-myostatin Shrna Constructs: The mRNA sequence of goat myostatin gene, retrieved from NCBI (accession no.AY436347) was used to design anti-myostatin shRNA constructs. The sequence was put in Dharmacon siDESIGN Center (www. dharmacon. com/sidesign/ siRNA) to generate different siRNA target sequences. Among all siRNA top four ranked siRNA were selected for experimental study [6]. The selected siRNAs were converted to shRNAs by using the Insert Design Tool for the *pSilencer*™ Vectors (http://www. ambion. com/ techlib/misc/psilencer_converter.html). The same miRNA (miR-26b) derived loop sequence (TTCAAGAGA) was chosen for all the shRNAs. This software tool generates siRNA into two linear nucleotide sequence, top and bottom strand. Few cytosine residues of the sense strand were changed to thymines which facilitate stability of vector in bacteria along with reduced interferon response [7]. For avoiding unwanted folding in antisense strand of the shRNA, the secondary structure (Fig. 1) was predicted by Vienna RNA Secondary Package (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Cloning of Shrna Inserts into Psilencer 4.1-cmv Neo Vector: The shRNA template oligonucleotides (top and bottom strand) were dissolved in nuclease-free water and finally a working solution of 1 µg/µl was prepared. These shRNA template oligonucleotides were annealed by gradual reduction in the temperature. For annealing the top and bottom strands, the annealing mixture was initially heated at 90°C for 3 min followed by gradual reduction in the temperature varying from 85°C to 45°C with 5°C decrement for 1 min each. Finally, the mixure was incubated for 1 hr at 37°C. The shRNA annealed oligonucleotides were ligated into *pSilencer* 4.1-CMV neo vector by T4 DNA ligase as recommended by the manufacturer (Fermentas). The ligated product was transformed into competent DH5α cells by using heat shock method. The transformed cells were grown over the LB-agar plate containing ampicillin (50µg/ml) as selectable marker. Single colonies were picked up and sub-cultured in fresh LB media. This was followed by the isolation of plasmid DNA.

Screening of Positive Clones: Screening of positive clones was done by PCR and restriction enzyme (RE) digestion. RE digestion reaction was carried out for plasmid containing the insert by *Hind*-III restriction enzyme at 37ºC in water bath for 6 hr to linearized the plasmid. The digested samples were resolved on 1% agarose gel in 0.5X TBE buffer (Fig. 2).

The clones were further confirmed by PCR using vector specific primer (Forward-5'AGGCGATTAAGTTGGGTA3' and Reverse-5'CGGTAGGCGTGTACGGTG 3') in programmable thermal cycler (ABI, USA) consisting of initial denaturation at

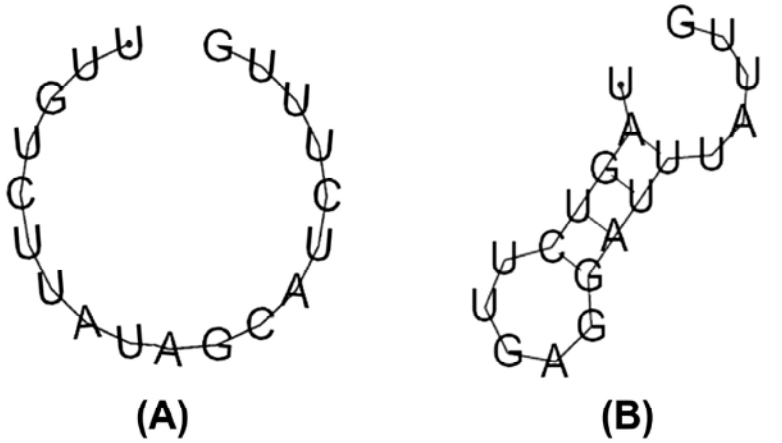

Fig. 1: Secondary structures of antisense strands of anti-myostatin shRNAs.
A - Antisense strand (sh1) with open circular structure.
B - Antisense strand (sh2) with free terminal ends.

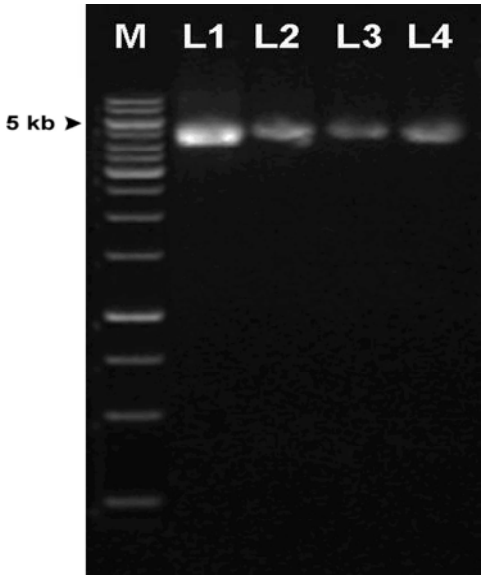

Fig. 2: Agarose gel electrophoresis (1.0%) showing the restriction enzyme analysis of *pSilencer*-anti-myostatin-shRNA constructs L1 to L4 are *Hind-III* digested sh1, sh2, sh3 and sh4 constructs

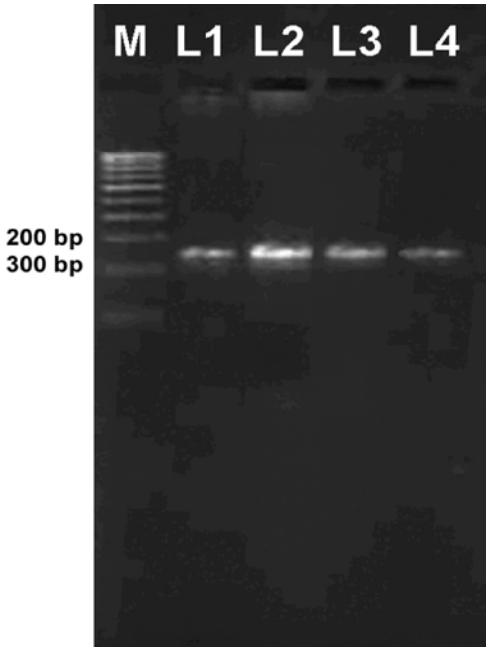

Fig. 3: Agarose gel electrophoresis (1.4%) showing the PCR amplified 245 bp shRNA along with vector sequence. L1 to L4 were the amplified sh1, sh2, sh3 and sh4 constructs from positive clones.

95°C for 5 min followed by 30 cycles, of denaturation at 95°C for 45 sec, annealing at 49°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. PCR products were resolved in 1.4% agarose gel electrophoresis (Fig. 3). After PCR amplification of shRNA sequences, the amplified products were subjected to gel purification for the sequencing.

Sequencing of the Constructs: The region of the plasmid containing the insert was sequenced using vector specific forward primer (5'AGGCGATTAAGTTGGGTA3') by automated DNA sequencer to detect any mutation (insertion or deletion) in the insert. A single base mutation in insert may lead to drastic changes in the results of the downstream applications like the study of the MSTN gene knockdown effects. The 245 bp PCR product having inserted anti-myostatin construct was used as a template for sequencing reaction. Nucleotide sequence obtained was aligned with standard vector sequence having shRNA (bottom strand) insert by the Megalign programme using Lasergene software (DNA STAR, USA).

RESULT AND DISCUSSION

Designing of Shrna Constructs: In the present study, the siRNAs were synthesized about 100 nucleotides downstream of the initiation codon (Table 1). The siRNAs synthesized against different regions of the same target mRNA showed different efficacy [8]. For optimizing siRNA-induced gene silencing several parameters like length, secondary structure, sugar backbone and sequence specificity of the siRNA duplex need to be analyzed. The first criteria for the design of RNAi molecules was provided by Elbashir *et al*. who suggested that siRNAs should not be created within the first 100 nucleotide of the AUG start codon mRNA as this region might bind RNA regulatory proteins or translation initiation complexes which could interfere with binding of RNA Induced Silencing Complex (RISC) to the target sequence [9]. Therefore, the targets in the 5' or 3' untranslated regions (UTRs) were avoided. It is imperative that all siRNAs constructed should be BLAST searched against EST libraries or mRNA sequences of the respective organism to minimize potential non-specific gene silencing. The BLAST results showed that the siRNAs were highly specific and without any significant similarity with the other EST libraries or mRNA sequences of the respective organism. The BLAST results clearly indicated that all four shRNA constructs were highly specific to myostatin gene in *Capra hircus*. Only myostatin gene showed 100% query coverage along with significantly low E-value, as desired in any BLAST result. Both of these parameters depict that all the four constructs are highly specific for MSTN gene.

Table 1: siRNA sequences selected to create shRNA sequences for targeting caprine myostatin gene with their 55bp long shRNA sequence in second row. (Nucleotides in italic show the siRNA sequence and in bold show the C to T change.)

S. No.	siRNA target sequence	Region	Start Position	GC %	Score
1	CAAAGATGCTATAAGACAA	ORF	219	32	90
	GATCC *CAAAGATGTTATAAGATAA* TTCAAGAGA TTGTCTTATAGCATCTTTG CT A				
2	CAATAAATCCTCAAGACTA	ORF	141	32	89
	GATCC *CAATAAATTCTTAAGACTA* TTCAAGAGA TAGTCTTGAGGATTTATTG TT A				
3	GCTCTAAGATACAACACAA	ORF	428	37	87
	GATCC *GCTTTAAGATACAATACAA* TTCAAGAGA TTGTGTTGTATCTTAGAGC TA A				
4	TAGCAGAAGTGCAAGAAAA	ORF	383	37	86
	GATCC *TAGCAGAAGTGTAAGAAAA* TTCAAGAGA TTTTCTTGCACTTCTGCTA GA A				

Any other gene or genome segment of *Capra hircus* did not show higher query coverage and score value. The E-value also increased abruptly, which indicated the significant difference among the more closely related blast search for each construct.

While designing shRNA constructs, 2-3 C nucleotides in the sense strand were changed to T's to facilitate stability in bacteria, increase shRNA efficiency and decrease possibility of self-targeting of the shRNA [7, 10]. Since the changes were not made in the antisense strand, the sequence of the antisense strand (guider strand) was not affected. The changes in the sense strand could result in destabilization of the duplex. Although our changes does not affect the 5' end of the antisense strand, it has been shown that siRNAs with low overall stability are less likely to be functional [6] and that excessive C to T changes might decrease silencing efficiency [7].

The GC content of the siRNAs designed in the present investigation ranged from 33 to 37% (Table 1). Most analyzed functional siRNAs have a low to medium (30% to 52%) GC content. Very low GC content may destabilize siRNA duplexes and reduce the affinity for target mRNA binding, whereas too high GC content may impede RISC loading and or cleavage-product release.

An important aspect regarding the most efficient designing of RNAi molecules is the secondary structure of antisense strand of shRNA. It might play an important role in the ability of these molecules to interact with target sequences. Several reports suggested that although the sequence of the RNAi molecule is important in gene silencing, yet if the molecule cannot have easy access to its target, then silencing would be limited [11-13]. In the present investigation, both open circular structure (Fig. 1A) and secondary structure with free terminal ends (Fig. 1B) were obtained. Lower free energies for the target region are correlated with open regions that contain a number of unpaired nucleotides and this was shown to correlate directly with siRNA efficiencies [14]. Moreover, Patzel *et al*. reported that it might not be the free energy of the secondary structure but the number of unpaired nucleotides at the 5′ and 3′ ends of the antisense strand that determines the efficiency of siRNAs [15]. Therefore, these constructs are expected to show good silencing efficiency on the basis of secondary structure of antisense strand. The constructs developed in the investigation can be further used in the *in vitro* (fibroblast or myoblast cell line transfection) or *in vivo* experiments on goat to study the knockdown effect of MSTN gene.

Cloning of shRNA Expressing Construct: The p*Silencer* 4.1-CMV neo vector was used in present study. This vector has modified Cytomegalomavirus (CMV) promoter which is considered to be a stronger promoter than the other common RNA *pol II* promoters used in mammalian expression vectors such as Simian virus-40 (SV40) and Rous sarcoma virus (RSV) [16]. *In vivo*, RNA *pol II* is primarily responsible for transcription of mRNA within the cell. The CMV promoter has the advantage of being highly active in a broad range of cell types and it does not interfere with other transcription events as may be the case with the RNA *pol III U6* and H1 promoters. This vector contains both ampicillin and neomycin resistance genes to enable antibiotic selection in bacteria and mammalian cells, respectively. Ampicillin selection was used to enrich the bacterial cells that were successfully transformed with vector by killing the cells that lacked the plasmid. Neomycin selection can be tried in further studies to select the clones containing recombinant plasmid in the eukaryotic system like fibroblast cell lines to develop the stably transfected cell lines.

Assessment and Selection of Positive Clones: The positive clones were first screened by restriction enzyme analysis of isolated plasmid. The plasmids of all the four constructs were isolated and subjected to RE digestion by *Hind*-III restriction enzyme at 37ºC in water bath for 6 hr to linearized the plasmid. The expected 4944 bp band was obtained on 1% agarose gel electrophoresis (Fig. 2).

Fig. 4 A (Sh1 construct)

```
R1 AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTAGCGGCCGCAT 82
R2 ---------------------------------------------------------------------------------- 0
R3 ----------------AAC--CAGGGTTTTCCCAGTCACAACGTTGTAAAACGACGGCCCGTGCCAAGATAGCGGCCGCAT 63

R1 ACAAAAAACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTAGCAAAGATGCTATAAGACAATCTCTTGA 164
R2 ----------------------------------------------AGCTTAGCAAAGATGCTATAAGACAATCTCTTGA 34
R3 ACAAATTACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTAGCAAAGATGCTATAAGACAATCTCTTGA 145

R1 ATTATCTTATAACATCTTTGGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCGTACACGCCTACCG 245
R2 ATTATCTTATAACATCTTTGG---------------------------------------------------------- 55
R3 ATTATCTTATAACATCTTTGGGAGCCACGGTTCACTAAACCAGCTCTGCTTATATAGACGTCCCACCGTACACGCCTACCG 226
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Fig. 4 B (Sh2 construct)

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R1 AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTAGCGGCCGCAT 82
R2 ---------------------------------------------------------------------------------- 0
R3 --------------------GCG-GGGTTTTCCCAGTCACGACGTTGTAAA-CGACGGCCAGTGCCAAGCTAGCGGCCGCAT 60

R1 ACAAAAAACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTAACAATAAATCCTCAAGACTATCTCTTGA 164
R2 ----------------------------------------------AGCTTAACAATAAATCCTCAAGACTATCTCTTGA 34
R3 ACAAAAAACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTAACA-TAAATCCTCAAGACTATCTCTTGA 141

R1 ATAGTCTTAAGAATTTATTGGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCGTACACGCCTACCG 245
R2 ATAGTCTTAAGAATTTATTGG---------------------------------------------------------- 55
R3 ATAGTCTTAAGAATTTATTGGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCG-ACACGCCTACCG 221
```

Fig. 4 C (Sh3 construct)

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R1 AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTAGCGGCCGCAT 82
R2 ---------------------------------------------------------------------------------- 0
R3 --------------------AGCATGATTT-CCCAGTC-CGACGTTGTAAA-CGACGGCCAGTGCCAAGCTAGCGGCCGCAT 59

R1 ACAAAAAACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTTAGCTCTAAGATACAACACAATCTCTTGA 164
R2 ----------------------------------------------AGCTTTAGCTCTAAGATACAACACAATCTCTTGA 33
R3 ACAAAAAACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTTAGCTCTAAGATACAACACAATCTCTTGA 141

R1 ATTGTATTGTATCTTAAAGCGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCGTACACGCCTACCG 245
R2 ATTGTATTGTATCTTAAAGCG---------------------------------------------------------- 55
R3 ATTGTATTGTATCTTAAAGCGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCG-ACACGCCTACCG 221
```

Fig. 4 D (Sh4 construct)

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R1 AGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTAGCGGCCGCAT 82
R2 ---------------------------------------------------------------------------------- 0
R3 ---------------------------------------------------------------------------------- 0

R1 ACAAAAAACCAACACACAGATCCAATGAAAATAAAAGATCCTTTATTAAGCTTTCTAGCAGAAGTGCAAGAAAATCTCTTGA 164
R2 ----------------------------------------------AGCTTTCTAGCAGAAGTGCAAGAAAATCTCTTGA 33
R3 GCAAATACCCAGCCCCCGGATCCA-TGAAAATAAAAGATCCTTTATTAAGCTTTCTAGCAGAAGTGCAAGAAAATCTCTTGA 81

R1 ATTTTCTTACACTTCTGCTAGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCGTACACGCCTACCG 245
R2 ATTTTCTTACACTTCTGCTAG---------------------------------------------------------- 55
R3 ATTTTCTTACACTTCTGCTAGGATCCACGGTTCACTAAACCAGCTCTGCTTATATAGACCTCCCACCGTACACGCCTACCG 162
```

Fig. 4: Sequence alignment report of all four shRNA constructs.
R1 is Standard shRNA sequence with vector
R2 is Standard bottom strand sequence
R3 is sequencing generated data for corresponding shRNA constructs.

Further confirmation of positive clones was done by PCR using vector specific primers. On agarose gel electrophoresis of PCR products, a desired band of 245 bp was obtained. This confirmed that the amplified product was shRNA along with vector sequence. All the four selected shRNA constructs were screened separately (Fig. 3).

Screening for Mutation Detection: It was found that multiple C to T mutations in the sense strand enhanced the silencing activity of the siRNA molecule produced from the shRNA [7]. However, mutations in the antisense strand resulted in almost no silencing of the target. An explanation for why the mutations could not be made in the antisense strand is that the 5′ end of this strand is used by RISC to "measure" the target point for cleavage (10 to 11 nt upstream of the 5′ region). Consequently, any mutations in this region could destabilize the bond formed between the antisense strand and the target in the RISC complex, which decreases the chances of cleavage [9, 17]. Conversely, mismatches in the sense strand can be tolerated to some extent.

SHRNAs are also known to be difficult to sequence due to their tight palindromic structure, thus insertions of nucleotides to form bulge loops makes it easier to sequence the constructs and it was demonstrated that these insertions have no effect on the activity of the RNAi molecule [7]. A further advantage of introducing mismatches in the sense strand is to prevent self-targeting of molecules i.e. targeting of the sense strand by the antisense strand instead of the target mRNA [18].

Any mutation in the antisense strand may result in almost no silencing of the target gene [7]. Mutation could destabilize the bond between the antisense strand and the target in the RISC complex and decrease the chances of cleavage [9,17]. Even a single nucleotide shift in target sequence might have a large effect on RNAi activity for example a single nucleotide shift resulted in 7000-fold higher half maximal inhibition (IC50) and a further single nucleotide shift restored the activity [15]. The sequencing of the constructs performed by using vector specific primers revealed the absence of mutation in any of the construct (Fig. 4).

In conclusion, four different shRNAs (sh 1, sh2, sh3 and sh4) against MSTN gene were designed and cloned in the pSilencer 4.1-CMV neo vector containing RNA *pol II* promoter. All the constructs were checked by PCR and direct sequencing for the confirmation of the positive clones. The sequencing of all the constructs showed the absence of mutations in any of the constructs. The constructs developed in the present investigation would be helpful in further experimental studies to investigate the knockdown effect in MSTN gene to increase the chevon production.

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