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Effect of myostatin gene silencing on the expression of extracellular matrix formative genes in caprine foetal fibroblast cells

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ABSTRACT

In vitro model for gene silencing using animal cells culture and use of these cells in production of transgenic animal could be an extremely valuable tool to mitigate the meat demand of the world. Myostatin had been identified as negative myogenic regulator that rolled as muscle mass enhancers when silenced. The present experiment was planned to test the effect of four 29-mer anti-MSTN shRNA constructs on silencing of myostatin gene in caprine foetal fibroblast cells and its effect on extracellular matrix formative genes in the caprine foetal fibroblast cells that are necessary for proliferation, differentiation, regulation and development of muscle in caprine. Experiment shows 55.1 to 91.5% of myostatin silencing in caprine foetal fibroblast cells and in myogenic gene expression; decorin shows down-regulation ranged from 78.46 - 89.03%. Whereas, follistatin found up-regulation (417.33 - 156.73%). The Pearson's correlation analysis revealed that there is negative correlation between myostatin and follistatin genes whereas decorin shows positive correlation. Result suggests that knock down of MSTN could be a promising approach to improve caprine musculatures and to establish important relationship between MSTN and extracellular matrix.

Key words: Caprine foetal fibroblast, Expression, Extracellular matrix, Myostatin, Silencing

Skeletal muscle development is a multistep regulatory process regulated by many genes, which governs the proliferation, determination, differentiation and fusion at appropriate time and place. Some genes and transcription factors related to muscle development are myostatin, decorin, follistatin Myf5, myogenin, MyoD etc. Myostatin acts as a negative regulator of skeletal muscle mass development. Myostatin, also known as GDF8 belongs to transforming growth factor-ß (TGF-ß) family that plays an essential role in skeletal muscle growth. Among all the myogenic genes, the central role was played by myostatin. Myostatin inhibited the progression of myoblasts from G1- to S-phase of the cell cycle through up-regulation of p21, the only Cdk2 inhibitor. Myostatin also inhibited myoblast differentiation by down-regulation of MyoD/myogenin expression (Luo *et al*. 2010). Inhibition of myostatin activity leads to increased muscle mass due to increased cell number as well as cell size (Tripathi *et al*. 2011).

Skeletal muscle fibers are surrounded by an extracellular matrix. The extracellular matrix is cell's secretory network of macromolecules and composed of glycoproteins, collagen, and proteoglycans, in which cells are embedded. Proteoglycans and glycoproteins have been suggested to play an important functional role in tissue differentiation (Bassols and Massague, 1988). Decorin is a leucine-rich chondroitin/dermatan sulfate proteoglycan that associates with both collagen types I and II. TGF-ß bounds to the decorin core protein, TGF-ß and decorin may be key players in a feedback regulatory loop. A specific functional interaction occurs between decorin and collagen in the extracellular matrix of soft tissues.

Follistatin was first isolated from ovarian follicular fluid. At late embryonic stages, follistatin is down-regulated in muscle, but expression resides in the connective tissue surrounding the muscles. The expression of myostatin is almost complementary to that of follistatin as it is expressed in a central domain of the dermomyotome, but not at the somite edges or in the hypaxial domain. Follistatin directly interacts with myostatin and it was found that follistatin blocked the inhibitory effect of myostatin (Zimmers *et al*. 2002) on the formation of differentiated muscle in a concentration-dependent manner (Amthor *et al*. 2004). Follistatin can almost completely prevent the effect of myostatin on Pax-3 and MyoD expression (Amthor *et al*. 2004). Follistatin consists of four domains, all work together for effective myostatin binding. Follistatin increases proliferation and delays differentiation of muscle precursors, dependent upon the presence of bone

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morphogenetic proteins (BMPs). Follistatin also modulate other inhibitors of muscle development, such as BMPs and activin. It emphasizes that follistatin could be a very potent molecule to combat muscle loss during dystrophies, muscle ageing, disuse or denervation.

RNAi is a post-transcriptional gene inhibitory method which usually utilizes short hairpin RNA (shRNA) expressed from plasmid or viral vectors to degrade the target. It is a specific and efficient gene silencing technique (Golding *et al*. 2006). It is gaining popularity because of its efficient gene silencing at low concentration and cost effectiveness (Jain *et al*. 2010). RNAi have been widely used in gene function analysis and the potentiality in therapeutics of diseases (Lares *et al*. 2010). RNAi mediated myostatin knockdown have some potential benefit for livestock owner, by increasing the live weight and carcass weight and improving the feed conversion efficiency, through a transgenic goat with double muscle phenotype (Tripathi *et al*. 2012). It is almost unclear that how myostatin knockdown, effect the expression of other muscle regulators in developing caprine foetal muscular progenitor cells.

In this study, the silencing of myostatin was evaluated through 29-mer based shRNA constructs in caprine foetal fibroblast cells of developing fetus about 45 days , as these cells have high prolificacy and might be going to serve as a donor cell in subsequent SCNT procedure for longer period of time. And we are also trying to evaluate *in vitro* myostatin silencing effect on expression of extracellular matrix formative genes along with the most efficient construct, which can be used to knockdown myostatin gene of goat origin to increase the meat production.

MATERIALS AND METHODS

Cloning of HuSH^{TM} pGFP-V-RS shRNA-29 expression vector: The complete mRNA sequence (1128bp) of Capra hircus myostatin gene (AY436347) was submitted to Origene Technologies, USA, to obtain four unique siRNA sequences. The siRNAs were converted to shRNAs by using the insert Design Tool for the pGFP-V-RS vector. This plasmid vectors contains both 5' and 3' LTRs of Moloney murine leukemia virus (MMLV) that flank the puromycin marker and the U6-shRNA expression cassette. A puromycin-N-acetyl transferase gene was located at downstream of the SV40 promoter, resulting in resistance to the antibiotic puromycin. The shRNA expression cassettes consists of a 29 nt target-gene-specific sequence, a 7 nt loop, and another 29 nt reverse comple-mentary sequence, all under the control of the human U6 promoter. A termination sequence (TTTTTT) is located immediately downstream of the second 29 nt reverse complementary sequence to terminate the transcription by RNA Pol III. The pGFP-V-RS vector contains the pCMV driven tGFP gene which expresses tGFP protein constitutively in mammalian cells. This feature makes it possible to monitor the transfection efficiency by observing the green fluorescent from the cells emitted under fluorescent microscope. The bacterial selection marker in this vector is Kanamycin. For

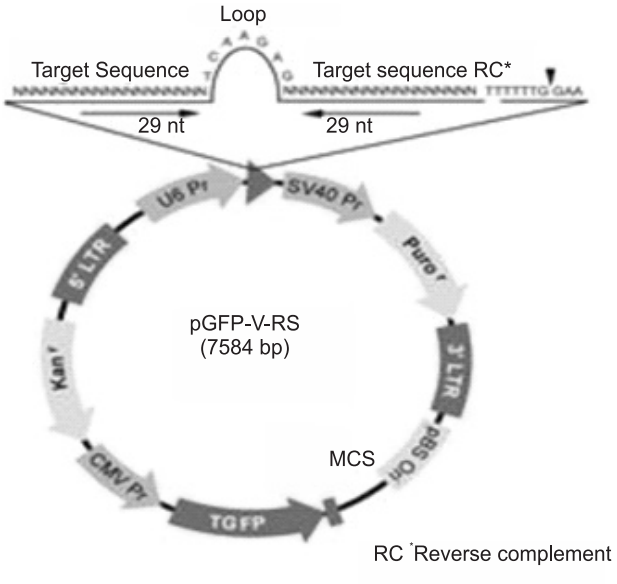

Fig. 1. Map of shRNA cloning pGFP- V- RS vector.

increasing stability of the vectors in bacteria, reducing interferon response, and to simplify sequencing some C nucleotides in the sense strand were replaced by T as suggested by Miyagishi *et al*. (2004). The Vienna RNA Secondary Package (http://www.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) was used to predict the secondary structure of the antisense strand of the shRNA. Insertion of the shRNA cassette into the vector destroys the BamHI restriction site by changing the sequence from GGATCC to GGATCG. Along with these synthesized oligos, manufacturer's supplied scrambled shRNA oligos were also ligated to pGFP-V-RS vector and used as negative control in the experiment.

The ligated product was transformed into competent DH5á cells by using heat shock method at 42°C for 45 sec. The transformed cells were grown on LB-agar plate containing nalidixic acid (30 mg/ml) and kanamycin (50 mg/ml). Screening of positive clones was done by colony PCR. In colony PCR, the shRNA along with vector sequence was amplified by vector specific primers F-AGGGCCTATTTCC-CATGATTC and R-TCCACACCC-TAACTGACACAC and the product was visualized on 2% agarose gel electrophoresis. The host cells harboring the positive clones were sub cultured two times in LB media containing nalidixic acid (30 mg/ml) and kanamycin (50 mg/ml). This was followed by the isolation of the plasmid DNA by midi plasmid kit.

Cell culture: Aborted caprine foetuses/slaughter house foetuses (about 45 days) were collected from slaughter house according to the guidelines and approval of Institutional ethics committee and primary caprine foetal fibroblast cell culture had been established in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum (FBS) (Gibco) at 38.5 °C and 5% carbon dioxide (CO_2) for first four days and foetal fibroblast cells were isolated further in 10% FBS. For medium change and subsequent passing by, 0.25% trypsin–EDTA, DMEM

supplemented with 10% fetal bovine serum was used.

Transfection of foetal fibroblast: Fibroblast cells were grown and after third passage 3×10^5 cells were seeded in six well plates and cultured in growth medium without antibiotics to achieve greater than 80% confluence on the day of transfection.

For transfection, 2µg of the plasmid vector containing 29-mer shRNA expression cassettes for each construct was used per well of cells. TurboFectin^{TM} 8.0 was used as the transfection reagent in a ratio of 2µg of DNA: 6µl of TurboFectin^{TM} 8.0 according to the manufacturer's instructions. Transfection was determined using green fluorescent protein expressing by the construct in the cells observed under fluorescent microscope after 48 hours of transfection. As controls, non-transfected and scrambled shRNA transfected cells were used.

Transfected foetal fibroblast cells with selectable antibiotic puromycin: The transfected cells were trypsinized by 250 µl trypsin-EDTA solution in each well of a 6 well plate by standard procedure. These transfected cells were seeded in 25 cm^2 flask contain puromycin 500 ng/ml of growth medium. After 48 hrs of antibiotic selection, the transfected cells were used for total RNA extraction.

Total RNA extraction and cDNA synthesis: Total RNA was isolated 48 h after transfection using Trizol (Invitrogen) according to the manufacturer's instructions. The quality of RNA was checked by Nanodrop ND–1000 spectrophoto meter. The RNA samples were then treated with DNaseI (Fermentas) for removal of possible genomic DNA contamination. The cDNA was synthesized using RevertAid ^{TM} MMLV RT enzyme. A total concentration of 286 ng RNA was used for cDNA preparetion using random hexamer primers.

Real-time RT-PCR analysis: Gene specific primers were designed using Primer 3 (http://frodo.wi.mit.edu/primer3/ Table 1) from the submitted sequence of NCBI. The mRNA expression levels of targeted myogenic genes and GAPDH was quantified in real-time PCR using SYBR Green chemistry following the manufacturer's protocol. Tripathi *et al.* (2011) have identified the primer pair 4.

All PCR reactions were performed in triplicates with Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control to normalize the amount of sample RNA in optical 96 well plates. Amplification was carried out in a final reaction volume of 10 µl containing 5µl SYBR Green PCR master mix, 1 µl of each gene specific forward and reverse primer (10 pmol), 2 µl of cDNA template and 1 µl nuclease free water. Thermal cycling condition comprised of initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec, primer annealing and extension at 60°C for 1 min. At the end of each run, a melt-curve analysis (95°C for 15 sec, 60°C for 1 min, an increase of 0.5°C/5 sec until 95°C) was performed to assess the specificity of the amplification. The specificity of amplification was verified by checking for correct size of amplification products on 2% agarose gel, and evaluation of melting curve during real-time PCR.

The ΔΔCT (Livak and Schmittgen 2001) method was used to determine the relative amounts of RNA transcripts for the genes of interest. GAPDH was tested and selected as the most stably expressed control gene for normalization. A real-time PCR mixture containing all components except template was used as a negative control. For relative quantification by the comparative C_T method, values were expressed relative to a reference sample (mock transfected fibroblast cells). The C_T for the target gene and the C_T for the internal control were determined for each sample and the calibrator. The expression of selected genes was calibrated by that of the reference gene, GAPDH, at each time point and converted to the relative expression (fold of expression), as follows

$$\text{Fold of expression} = 2^{\Delta\Delta C_T}$$

where, ΔC_T = average C_T of target gene – average C_T of endogenous control (GAPDH)

ΔC_T = average ΔC_T of target sample – average ΔCT of calibrator sample (fibroblast)

Statistical analysis: All data are presented as Mean ± SE; between different samples the student's t-test was performed to identify significant changes. All experiments were repeated in triplicate. Pearson correlation was calculated using the Excel Analysis ToolPak. The level of significance chosen to determine whether treatments were significantly different to controls is $P < 0.05$ and 0.01.

RESULTS AND DISCUSSION

Evaluation of anti-myostatin shRNA constructs: In this study four HusH^{TM} 29-mer anti-myostatin shRNA constructs were used. The shRNA consists of a sense strand, an antisense strand and a short loop sequence between the

Table 1. Gene specific primers

Set	Primer Name	Length	Primer sequence (52 -32)	Amplicon Size (bp)	NCBI Accession no.
1.	GAPDH-F	17	GGCGCCAAGAGGGTCAT	69	AJ431207
	GAPDH-R	19	GTGGTTCACGCCCATCACA		
2.	MSTN-F	21	TTTGGCAGAGCATTGATGTGA	100	AY436347
	MSTN-R	25	TGACCATTCTCATCTAAAGCTTTGA		
3.	Follistatin F	19	GGACTTCAAGGTTGGCAGA	80	HM138666
	Follistatin R	21	CACAGACAGGCTCCTCAGACT		
4.	Decorin F	19	CATTTGCTCCTCTGGTGAA	134	FJ966918
	Decorin R	20	AGACTTTCGCACTTTGGTGA		

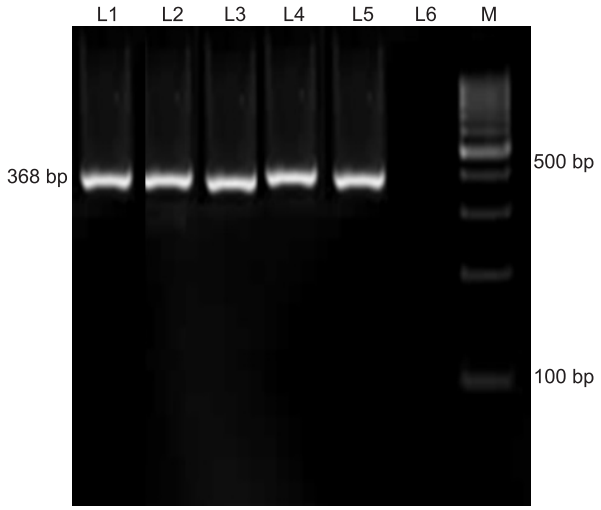

Fig. 2. Colony PCR product in 2% agarose gel electrophoresis. L, Lane; L1, shA; L2, shB; L3, shC; L4, shD; L5, shS; L6, NTC; M, ladder.

sense and antisense fragment. The antisense strands were checked for the presence of any secondary structure. After the transformation of these constructs in *Escherichia coli DH5á* cells, transformed cells were used for colony PCR and a clear 368 bp PCR product was seen on 2% agarose gel electrophoresis (Fig. 2).

Caprine fetal fibroblast cell culture establishment: The caprine foetal fibroblast cells the prominent growth of cells i.e. splitting was noticed on next day (Fig. 3) after seeding of small tissue pieces into tissue culture flask passaging was carried out by adding 250µl 0.25% trypsin-EDTA to more than 80–90% confluent tissue culture flask and incubating it for 2–3 min at 38.5°C followed by centrifuge at 3,000 rpm for 3 min.

Determination of transfection by visualizing green fluorescence: A visual demonstration of GFP marker gene is the proof of transfection and it is the quickest way to show the presence of shRNA constructs inside the cells. The results of gene silencing efficiency of shRNA constructs

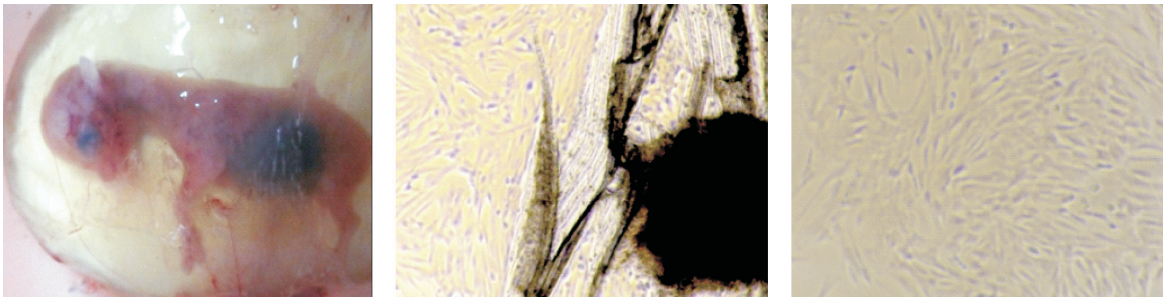

Fig. 3. Culture of Caprine foetus fibroblast cells. A, Caprine foetus in the amniotic fluid; B, Tissue piece showing splitting of cells (100×); C, Caprine fetal fibroblast cells after third passage (100X).

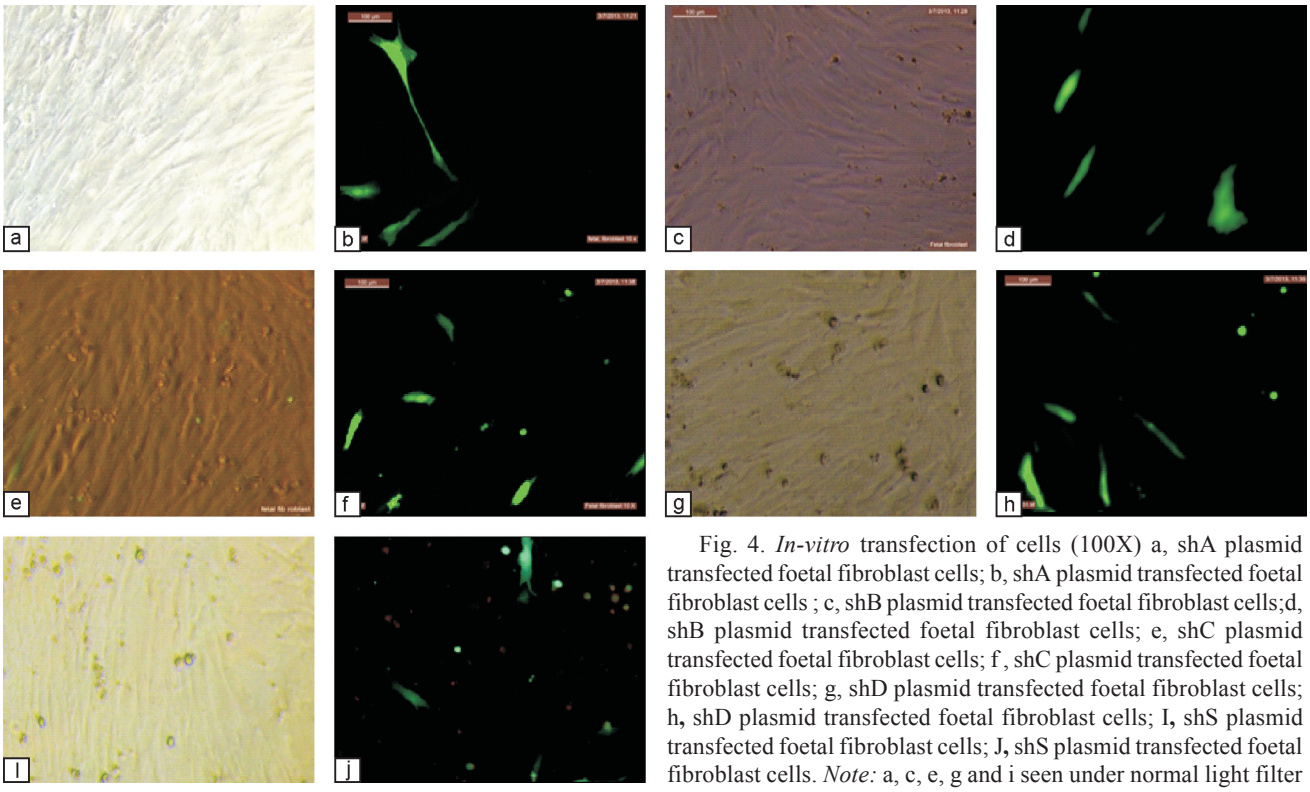

Fig. 4. *In-vitro* transfection of cells (100X) a, shA plasmid transfected foetal fibroblast cells; b, shA plasmid transfected foetal fibroblast cells ; c, shB plasmid transfected foetal fibroblast cells;d, shB plasmid transfected foetal fibroblast cells; e, shC plasmid transfected foetal fibroblast cells; f , shC plasmid transfected foetal fibroblast cells; g, shD plasmid transfected foetal fibroblast cells; h**,** shD plasmid transfected foetal fibroblast cells; I**,** shS plasmid transfected foetal fibroblast cells; J**,** shS plasmid transfected foetal fibroblast cells. *Note:* a, c, e, g and i seen under normal light filter and b, d, f, h and j seen under GFP filter.

by real-time PCR can only be validated when there is a proof of significant transfection (Stewart *et al.* 2008). Hence, in the present study, the p-GFP-v-RS vector was used which contains a reporter gene, GFP. This pCMV driven tGFP gene expresses tGFP protein constitutively in mammalian cells. In the present study, all the constructs namely, shA, shB, shC and shD showed good transfection (Fig. 4).

Myostatin silencing and myogenic genes expression: For myostatin silencing by HusH^{TM} shRNA constructs in caprine foetal fibroblast cells and the transfection complex was formed. The transfection was performed in triplicate for all four constructs showed significant myostatin reductions ($P<0.01$). Among all constructs shC showed maximum reduction of myostatin (91.5%) followed by shD (79.7%), shA (74.7%) and shB (55.1%) in reference to mock control. Previously fibroblast cell lines have been successfully used to study MSTN expression by many workers (Stewart *et al.* 2008, Jain *et al.* 2010, Tripathi *et al.* 2011). Jain *et al.* (2010) reported the silencing of 22–92% MSTN gene in caprine fibroblast cell line by four different 21 mer pSilencer 4.1-CMV shRNA constructs. Tripathi *et al.* (2012) reported 7–46% of myostatin silencing in caprine myoblast cells by six different shRNA constructs.

In the present experiment the level of expression of decorin showed down- regulation that ranged from 78.46 – 89.03% in respect to the mock. It was observed that shA transfected caprine foetal fibroblasts had high down-regulation (89.03%), followed by shC (83.54%), shD (79.01%) and shB (78.46%). Bassols and Massague (1988) reported increase in TGF-ß up-regulation synthesis of decorin. Similar finding was also reported by McFarland *et al.* (2006).

In this study, it was found that the follistatin gene expression was up-regulated. The maximum up-regulation was found for shC construct (317.33%), followed by shD (269.26%), shA (129.8%) and shB (56.73%). The follistatin gene expression after MSTN silencing was

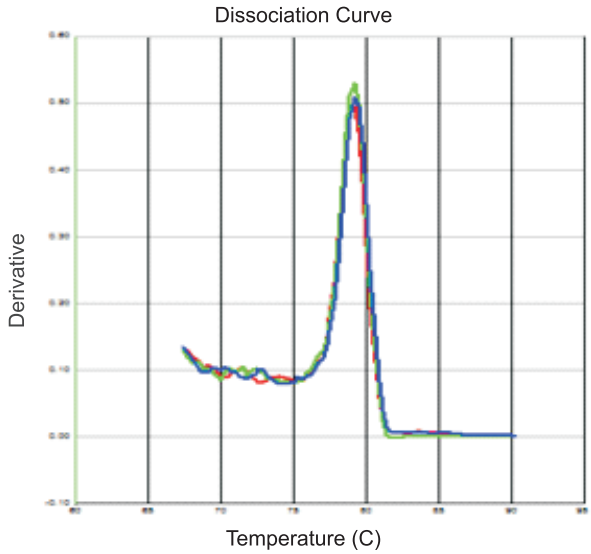

Fig. 5. Dissociation curve GAPDH.

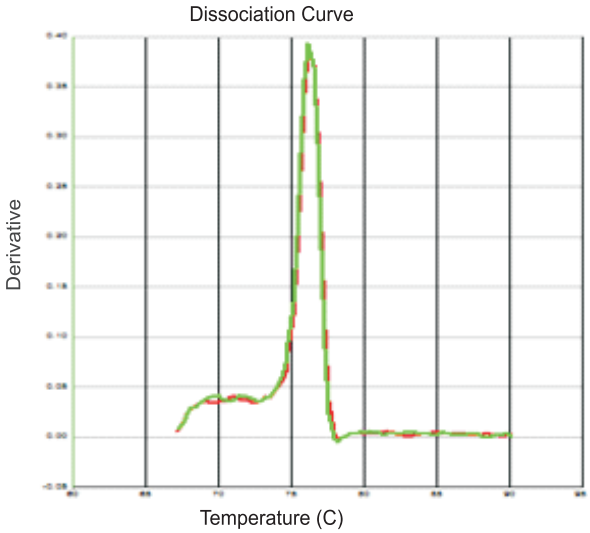

Fig. 6. Dissociation curve myostatin.

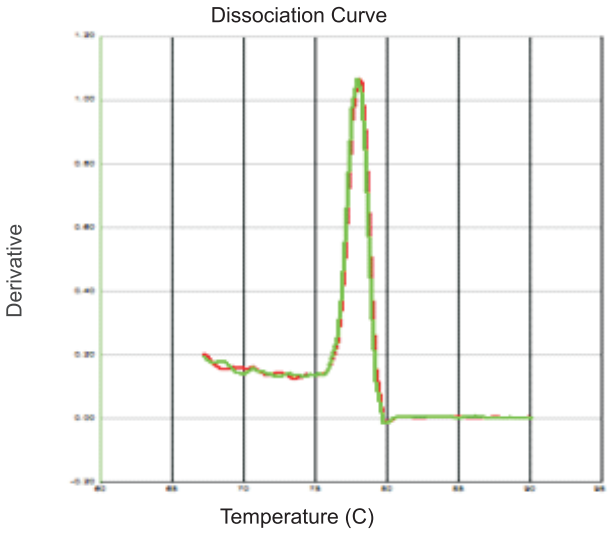

Fig. 7. Dissociation curve decorin.

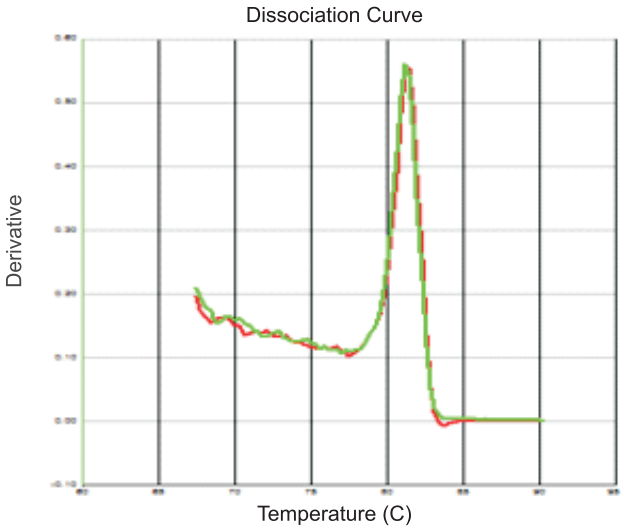

Fig. 8. Dissociation curve follistatin.

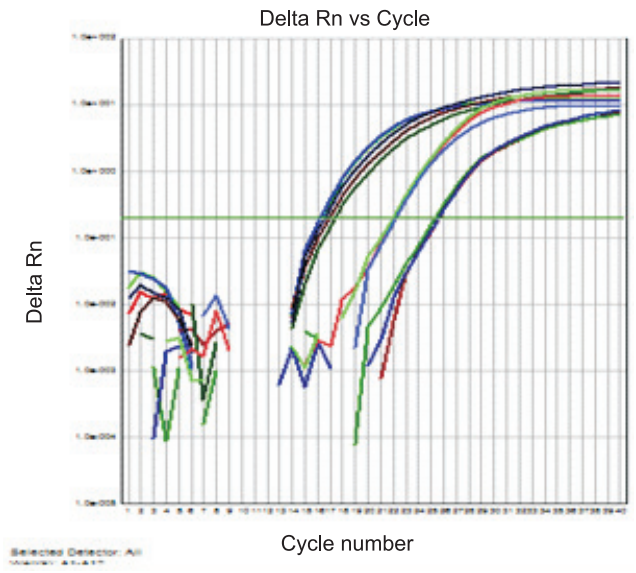

Fig. 9. Amplification plot of GAPDH, myostatin, decorin, follistatin.

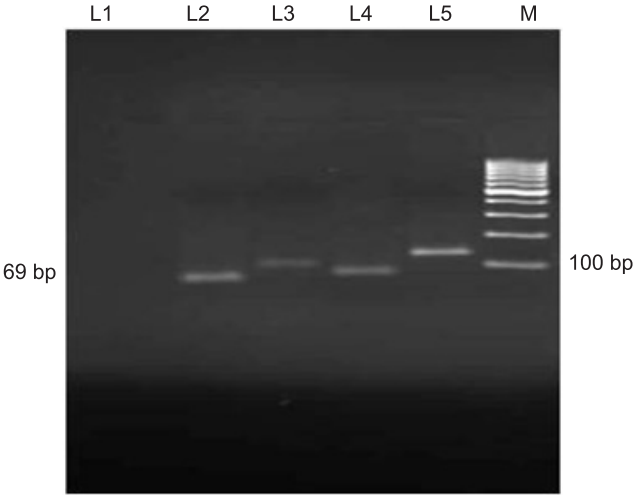

Fig. 10. Agarose gel electrophoresis (2%) showing real-time PCR amplified products. L1, NTC; L2, GAPDH; L3, myostatin; L4, follistatin; L5, decorin; M, 100 bp ladder .

almost similar to the expression pattern reported by Amthor *et al.* (2004).

Pearson's correlation was applied to establish the significant correlation in myostatin and extracellular matrix formative genes for four shRNA constructs**.** Results showed positive correlation between myostatin and decorin whereas follistatin shows negative correlation in respect to all constructs.

Genes	Constructs			
	shA	shB	shC	shD
Decorin	0.987	0.638	0.922	0.984
Follistatin	–0.740	–0.560	–0.970	–0.530

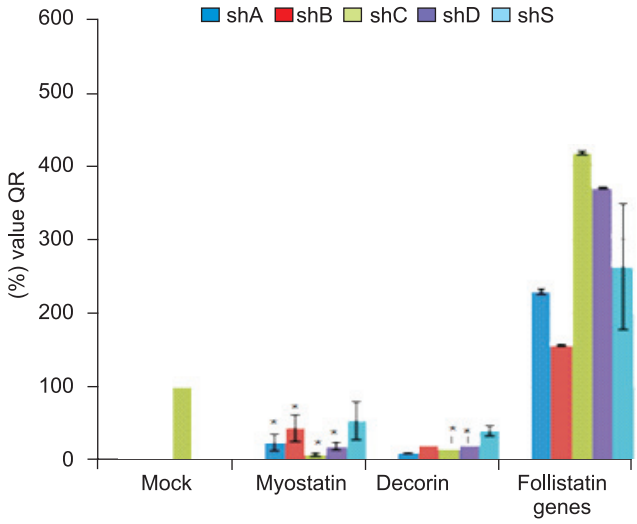

Fig. 11. Expression profile of genes in shRNA transfected caprine fetal fibroblast cells. * (P< 0.01).

The present study provides good evidence that shRNA mediated gene silencing is a powerful method for loss-of-function studies in cultured caprine foetal fibroblast cells. This level of MSTN silencing due to the fact that the unstructured antisense strand, or having several free nucleotides at both ends of the strands, mediates the silencing, the shC construct showed highest silencing, it might be related to fact that the presence of cytidine nucleotide and 5' and 3' ends accessibility in both the end.

The result of the present experiment revealed (Fig 5–11) that there is a strong relationship between the myostatin and follistatin. In the normal cell regulatory pathways the myostatin might be overpowering the follistatin and maintained homeostasis. When myostatin effect is being abolished the expression of follistatin might get upper hand. Generally decorin expression increases with increase in TGF-ß, when the expression of myostatin is decreased its activity is also suppressed resulting in cellular proliferation, extracellular matrix formation, hyperplasia and hypertrophy of the muscular tissue and increases the muscle mass. But we did not observe a noticeable liner relationship between myostatin down-regulation with follistatin up-regulation and decorin down-regulation.

On the basis of results shC construct would be helpful in the development of stably transfected (anti-myostatin shRNA transformed) cell line which may be used as a donor cell for the creation of goat with enhanced muscle growth, through Somatic Cell Nuclear Transfer (SCNT) technology.

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