

# **AAV-2 VECTOR INTEGRATE INTO THE OVINE MYOBLAST GENOME RANDOMLY AND PROMOTE DIFFERENTIATION AND PROLIFERATION VIA FOLLISTATIN OVER-EXPRESSION OF ERK1/2 AND AKT SIGNALING PATHWAYS**

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## **ABSTRACT**

The aim of our study was to investigate effect of FST over-expression by using AAV serotype 2 (AAV 2) vector on ovine primary myoblast (OPM) differentiation and proliferation. Primary myoblast cultures were obtained from 60-day-old sheep fetuses. Western blot confirmed that AAV2 could successfully express FST protein in transduced primary myoblast cells. Southern blot results demonstrated that AAV vectors integrated at apparently random genomic sites and promoted the transgenic myoblast proliferation and differentiation. The results suggested that the AAV system could be used to generate transgenic meat sheep in the future.

## **INTRODUCTION**

These features give the AAV System a superior biosafety rating among gene delivery and expression vectors of viral origin. AAV vectors transduce a variety of somatic tissues, including skeletal muscle, without eliciting an immune response in mice (1). Recent reports

have indicated that AAV vectors are capable of integrating the follistatin gene into the host chromosome and facilitating long-term transduction (2, 3). Follistatin (FST) has been demonstrated to be a potent antagonist of other members of the TGF- $\beta$  family, including myostatin (4). Indeed, over-expression of follistatin by transgenic approaches in muscle has been shown to increase muscle growth *in vivo*, and a lack of follistatin results in reduced muscle mass at birth (5). Several studies have also shown that FST is capable of controlling muscle mass through pathways independent of the myostatin signaling cascade (6). Myostatin (MSTN) negatively regulates myoblast proliferation through the activation of Smad, Akt, p38MAPK and p21 pathways (7-10). Antagonists of MSTN have shown considerable promise for enhancing muscle mass and strength. MSTN inhibits proliferation and differentiation of myoblasts, limiting the growth rate and muscle mass in mammals (6). Recent studies have highlighted the potential benefit of inhibiting MSTN, which results in a double muscle phenotype in MSTN-deficient cattle (11) and MSTN-knockout mice (12). In particular, because sheep are an economically important animal, breeding double muscle sheep is of high economic value. However, AAV-mediated FST gene transfer has not been reported in sheep, whereas there are several reports of FST gene transfer in sheep by other vectors, such as lentiviral vectors (13). Using of AAV vectors to produce transgenic animal can increase biosafety rating for human. The objective of the current study was to use a recombinant AAV serotype 2 (rAAV2) carrying follistatin to explore the effects of FST on ovine primary myoblast (OPM) differentiation and proliferation *in vitro*. In the present study, we tested the hypothesis that an AAV-2 virus carrying the FST gene is capable of inducing ovine myoblast differentiation and proliferation *in vitro*. Our results demonstrated that AAV-2 vector can integrate at apparently random genomic sites and promote the myoblast proliferation and differentiation.

## **MATERIALS AND METHODS**

### ***Plasmid Construction***

A complete open reading frame (ORF) of 1035 base pairs of the ovine FST gene was amplified via PCR from full-length cDNA using the forward primer 5'-AAGAATTCCCTCAGGATGGCCCGTCCTA-3' (P1) containing an EcoRI site (underlined) and the reverse primer 5'-GCTCGAGGGTTTTCCACTCTAGAATGGA -3' (P2) containing an XhoI site (underlined). The primers were designed based on the availability of ovine sequences (Gene Bank Accession No. KF833357). The PCR products and the pAAV-IRES-GFP vector (Agilent Technologies Company, La Jolla, CA) were digested with EcoRI and XhoI (Fermentas, Life Sciences, Thermo Fisher), recovered through agarose gel electrophoresis, and then ligated by T4 DNA ligase (New England Biolabs, Beverly, MA). New recombinant plasmids, which consisted of a CMV promoter and a FLAG tag, carrying the ovine FST gene were termed pAAV-CFS-FLAG.

### ***Packaging AAV vectors***

AAV particles were produced by co-transfection of the recombinant pAAV-CFS-FLAG vector (20 µg) with two helper plasmids (20 µg each of pAAV-RC and pHelper) into HEK 293 cells. For purification of AAV particles, a ViraBind™ Adeno-Associated Virus Purification Mega Kit (Cell Biolabs, INC, San Diego, CA, USA) was used according to manufacturer's protocol.

### ***Ovine primary myoblast culture and differentiation***

Primary myoblast cultures were obtained from 60-day-old sheep fetuses, as previously described (14).

### ***Western blot analysis***

Total protein was extracted quantified using a Nuclear Extraction Kit from the normal myoblast cells and the transduced myoblast cells to determine MSTN and FST protein

expression and Total protein was obtained from hind limb fetal skeletal muscle as a control. A total of 20 µg protein was separated via SDS-PAGE (12%) and transferred to a nitrocellulose membrane via electro-blotting. The gels run under non-reducing conditions. SDS-PAGE and western blot transfer were performed using standard techniques. Horseradish peroxidase activity was detected using ECL plus Western blotting detection reagents (RPN2132, Amersham Biosciences) and quantified by densitometry by the GelQuantNET software, and normalized to GAPDH.

### ***Southern blot analysis***

Genomic DNA from transgenic and non-transgenic myoblast was purified and aliquots of DNA (5 µg) were digested by two restriction enzymes EcoRI and HindIII. To remove single-stranded rAAV genomes and concatamers, DNA was treated with ATP-dependent exonuclease (4 U per µg; Plasmid Safe; Epicenter) at 37°C overnight after enzyme digestion. Then, the DNA was resolved on 0.8% agarose gel and then transferred onto a positive-charged nylon membrane with a vacuum-transfer system. The probe was prepared by PCR amplification using the forward primer 5' - CTGCCCGCCATGGAGATCGAGT -3' and reverse primer 5' - GGCATCTGCATCCGGGGTCTTG -3' designed based on the GFP sequence. Hybridization and detection were performed by using a digoxigenin (DIG) high prime DNA labeling and detection starter kit II (Roche Molecular Biochemicals, Indianapolis, IN, USA) according to the manufacturer's instructions.

### ***Cell staining and determination of fusion percentage***

Cultures were stained and examined microscopically to determine the percentage of nuclei in myotubes, as previously described (15). Fusion percentage was assessed by determining the ratio of the number of myotube nuclei to total nuclei.

### ***Cell proliferation assay***

After the incubation period, the cell proliferation assay was performed by adding 20  $\mu$ l of the Cell-Counting Kit-8 (CCK-8) reagents (Dojindo Molecular Technologies, Maryland, USA) to each well of the plate for 2 h. Finally, the absorbance at 450 nm was measured using a Spectra Max M5 microplate reader (Molecular Devices, CA, USA).

### ***Producing positive transgenic myoblast cells***

GFP-positive cell sorting from live population cells was performed on a FACS (MoFlo® Astrios™) system. The sorted positive cells were stable transfected cells.

***Statistics***— The data are expressed as the mean  $\pm$  SEM. The experiments for RT-qPCR were repeated in replicates of eight but samples were repeated in triplicate for cell cycle and western blot analysis. One way ANOVA was performed to identify significant changes between different groups. Student's t- test was performed to identify significant changes using SPSS software (version 13.0). Differences are reported at two significance levels, 0.05 or 0.01.

## **RESULTS**

### ***Myoblast proliferation is increased by follistatin over-expression***

As shown in Fig. 1, the optical density (at 450 nm) was significantly increased in the transduced OPMs cultured in GM media after 48, 72 and 96h compared with the control, indicating that FST significantly increased proliferation ( $P<0.01$ ). However, the FST protein induced the growth of myoblasts in a time-dependent manner with half-maximal induction occurring at the 72h time point (Fig. 1). These data indicated that when OPM cells were incubated in GM with the AAV-CFs-FLAG virus for 96 h, there was a steady increase in cell number compared to the control (Fig. 1). The results strongly suggested that AAV-CFs-FLAG-expressing FST induced a significant effect on cell myoblast proliferation, suggesting that it is involved in modulating myoblast proliferation.

### ***Follistatin over-expression promotes ovine myoblasts differentiation***

Table I shows the fusion percentage average in myotubes in ovine transduced myoblast. The average percentage of nuclei observed in myotubes transduced with AAV-CFS-FLAG was 47.11%, which was significantly greater than the 32.05% observed with non-transduced cells (Figs 2;  $P < 0.01$ ,  $n = 10$ ). These findings suggest that the addition of ovine follistatin to differentiation medium resulted in more nuclei contributing to myotube formation than non-transfected myoblasts. These results clearly suggested that AAV-CFS-FLAG expressing follistatin increased the differentiation of primary myoblasts.

### ***FST overexpression induces phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and AKT***

Western blot analysis was performed to determine effect of follistatin over-expression on the phosphorylation of MAPKs such as ERK1/2 and Akt in proliferation condition. Total protein was isolated after 96 h from the OPM transduced with AAV-CFS-FLAG and the non-transduced OPMs (control). As shown in Fig. 3, follistatin overexpression induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 and AKT. The amount of ERK 1 and ERK 2 phosphorylation after induction time of 96h were increased 1.7 and 2.3-fold, respectively.

### ***AAV vector integrates into the myoblast genome***

The GFP probe was hybridized with genomic DNAs from ovine transgenic and non-transgenic cells (Fig. 4). The southern blot confirmed stable integration of the transduced AAV-CFS-FLAG into the ovine genome for transgenic myoblast cells. Moreover, these results indicate that in transgenic cells the transposition occurred as multiple copy integration in the different genomic site. As shown in Fig. 4, southern blot results indicated two and three bands for TC1 and TC3 and also four and five bands for TC4 and TC2, respectively.

## DISCUSSION

*FST over-expression promotes proliferation through activation of ERK1/2 and Akt pathways*—We performed densitometric analysis of the data obtained from multiple experiments (n=3) in proliferation condition (Fig. 3). Follistatin over-expression by AAV2 induced the pERK1 and pERK2 levels 1.7 and 2.3-fold ( $P < 0.01$ ), respectively. As expected, ERKs (mainly p42 MAPK) were robustly activated in transduced cells (Fig. 3, top, lane 1). These results suggest that Follistatin over-expression induced cell proliferation via the activation of the ERK pathway.

In exploring the signaling mechanism, we focused on Akt, as it is a well-acknowledged critical signaling node within the cells under both physiological and pathological conditions and it plays a pivotal role in cell survival. It is known that activation of Akt is critical for cell survival (19). Here we assessed Akt activation by immunoblotting with an anti-activated Akt antibody (pAkt) in ovine myoblast cells under proliferation conditions as described in Fig. 5. Akt was robustly activated in the transduced cells (Fig. 3). Our findings indicated that follistatin regulated the ERK1/2 (18) and Akt/PKB signaling pathways (20). Our result showed that follistatin over-expression induced the phosphorylation of ERK1/2 and Akt, resulting in a significant promotion of ovine myoblast cell proliferation.

## CONCLUSIONS

Our results provide the first evidence that the AAV viral system can use to gene transfer in sheep. Our findings suggest that AAV vectors integrate at apparently random genomic sites and promote the transgenic myoblast proliferation and differentiation.

These results expanded our understanding of the regulation mechanism of FST in ovine primary myoblasts. Our findings demonstrated that FST promotes proliferation through activation of ERK1/2 and AKT pathways in OPMs under proliferating conditions.

## REFERENCES

- 1- Russell, DW.; Deyle, DR: Adeno- associated virus vector integration. *Current Opinion in Molecular Therapy* 2010, **11** (4): 442–447.
- 2- Haidet, AM.; Rizo, L.; Handy, C.; Umaphathi, P.; Eagle, A.; Shilling, C.; Boue, D.; Martin, PT.; Sahen, Z.; Mendell, JR.; Kaspar, BK: Long-term enhancement of skeletal muscle mass and strength by single gene administration of Myostatin inhibitors. *Proc Natl. Acad. Sci. USA* 2008, **105**: 4318–4322.
- 3- Kota, J.; Handy, CR.; Haidet, AM.; Montgomery, CL.; Eagle, A.; Mendell, JR.; Kaspar, BK: Follistatin Gene Delivery Enhances Muscle Growth and Strength in Nonhuman Primates. *Sci Transl Med* 2010, **1**(6): 1-17.
- 4- Amthor, H.; Nicholas, G.; McKinnell, I.; Kemp, CF.; Sharma, M.; Kambadur, R.; Patel, K: Follistatin complexes myostatin and antagonizes myostatin-mediated inhibition of myogenesis. *Devel Biol* 2004. **270**, 19–30.
- 5- Lee, SJ.; McPherron, AC: Regulation of Myostatin activity and muscle growth. *Proc Natl Acad Sci USA* 2001, **98**: 9306–9311.
- 6- Lee, SJ: Sprinting without Myostatin: a genetic determinant of athletic prowess. *Trends Genet* 2007, **23**: 475-477.
- 7- Han, DS.; Huang, HP.; Wang, TG.; Hung, MY: Transcription activation of myostatin by trichostatin A in differentiated C2C12 myocytes via ASK1-MKK3/4/6-JNK and p38 mitogen-activated protein kinase pathways. *J Cell Biochemistry* 2010, **111**: 564-573.
- 8- Langley, B.; Thomas, M.; Bishop, A.; Sharma, M.; Gilmour S, Kambadur, R: Myostatin Inhibits Myoblast Differentiation by Down-regulating Myo D Expression. *J. Biological. Chem* 2002, **277**: 49831–49840.
- 9- Philip, B.; Lu, Z.; Gao, Y: Regulation of GDF-8 signaling by the p38 MAPK. *Cell Signal* 2005, **17**: 365-375.

- 10- Trendelenburg, AU.; Meyer, A.; Rohner, D.; Boyle, J.; Hatakeyama, S.; Glass, DJ: Myostatin reduces Akt/TORC1/p70S6K signaling, inhibiting myoblast differentiation and myotube size. *American J Cell Physiol* 2009, **296**: C1258-70.
- 11- Grobet, L.; Martin, LJ.; Poncelet, D.; Pirottin D: A deletion in the bovine myostatin gene causes the double muscled phenotype in cattle. *Nature Genetic* 1997, **17**: 71–74.
- 12- Wagner, KR.; McPherron, AC.; Winik, N.; Lee, SJ: Loss of myostatin attenuates severity of muscular dystrophy in mdx mice. *Ann Neurol* 2002, **52**: 832–836.
- 13- Zhang, N.; Zhang, X.; Liu, M.; Tan, L: Ovine Follistatin gene expression and functional analysis of Follistatin domains. *Chin J Biotech* 2010, **26**: 1050–1056.
- 14- Hembree, JR.; Hathaway, MR.; Dayton, WR: Isolation and culture of fetal porcine myogenic cells and the effect of insulin, IGF-1, and sera on protein turnover in porcine myotube cultures. *Journal of Animal Science* 1991, **69**: 3241-3250.
- 15- Kirstin, G.; Gareth, EJ.; Ramya, T.; Diana, J: The effect of galectin-1 on the differentiation of fibroblasts and myoblasts in vitro. *Journal of Cell Science* 2002, **115**: 355-366.
- 16- Thomas, M.; Langley, B.; Berry, C.; Sharma, M.; Kirk, S.; Bass, J.; Kambadur, R: Myostatin, a Negative Regulator of Muscle Growth, Functions by Inhibiting Myoblast Proliferation. *J. Biological. Chem* 2000, **275**: 40235–40243.
- 17- Li, ZB.; Kollias, HD.; Wagner, KR: Myostatin directly regulates skeletal muscle fibrosis. *J Biol Chem* 2008, **283**: 19371-19378.
- 18- Johnson, GL; Lapadat, R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002, **298**: 1911–1912.
- 19- Manning, BD.; Cantley, LC: AKT/PKB signaling: navigating downstream. *Cell* 2007, **129**: 1261–74.

20- Song, G.; Ouyang, G.; Bao, S: The activation of Akt/PKB signaling pathway and cell survival. *J. Cell. Mol. Med* 2005,**9** (1): 59–71.

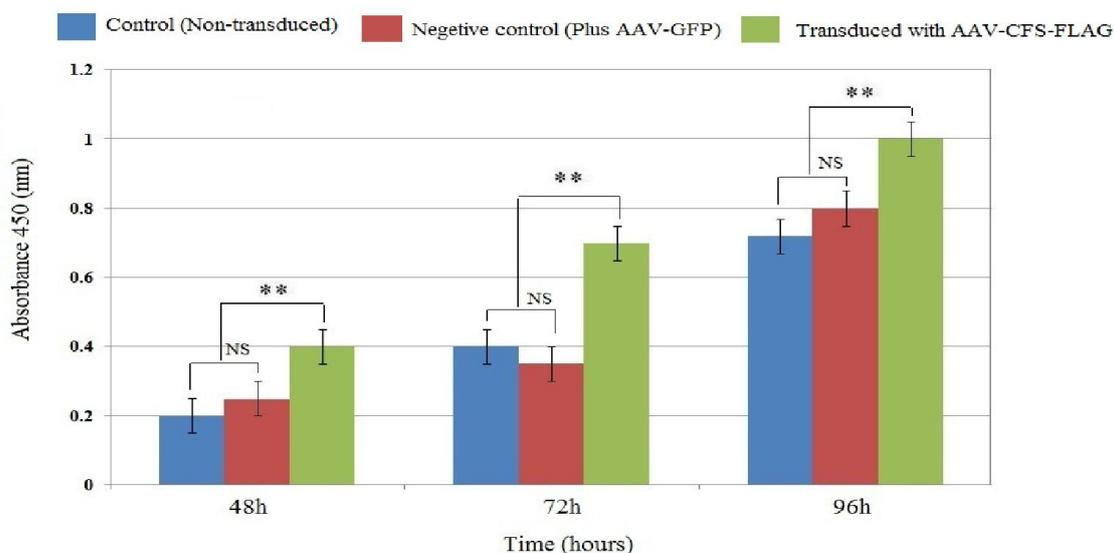


Fig (1) Induction of ovine primary myoblast proliferation by AAV-CFs-FLAG virus expressing FST protein in vitro. Absorbance (at 450 nm) was read at 48, 72 and 96 hours after plating in growth media without or with AAV-CFs-FLAG virus. Data were analyzed by one-way ANOVA (\*\* P < 0.01, n=8, with non-transduced and negative control).

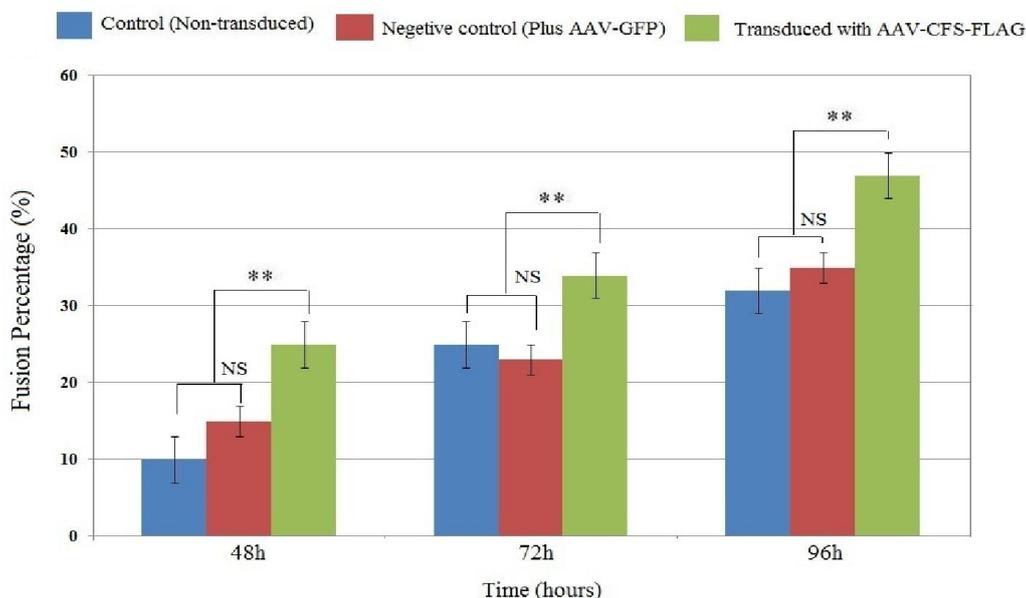


Fig (2) Induction of ovine primary myoblast differentiation by AAV-CFS-FLAG in vitro. Fusion percentage was calculated for the cells cultured in differentiation media for 48, 72 or 96 hours.

96 h. Data were analyzed by one-way ANOVA (\*\* P < 0.01, n=10, with non-transduced and negative control).

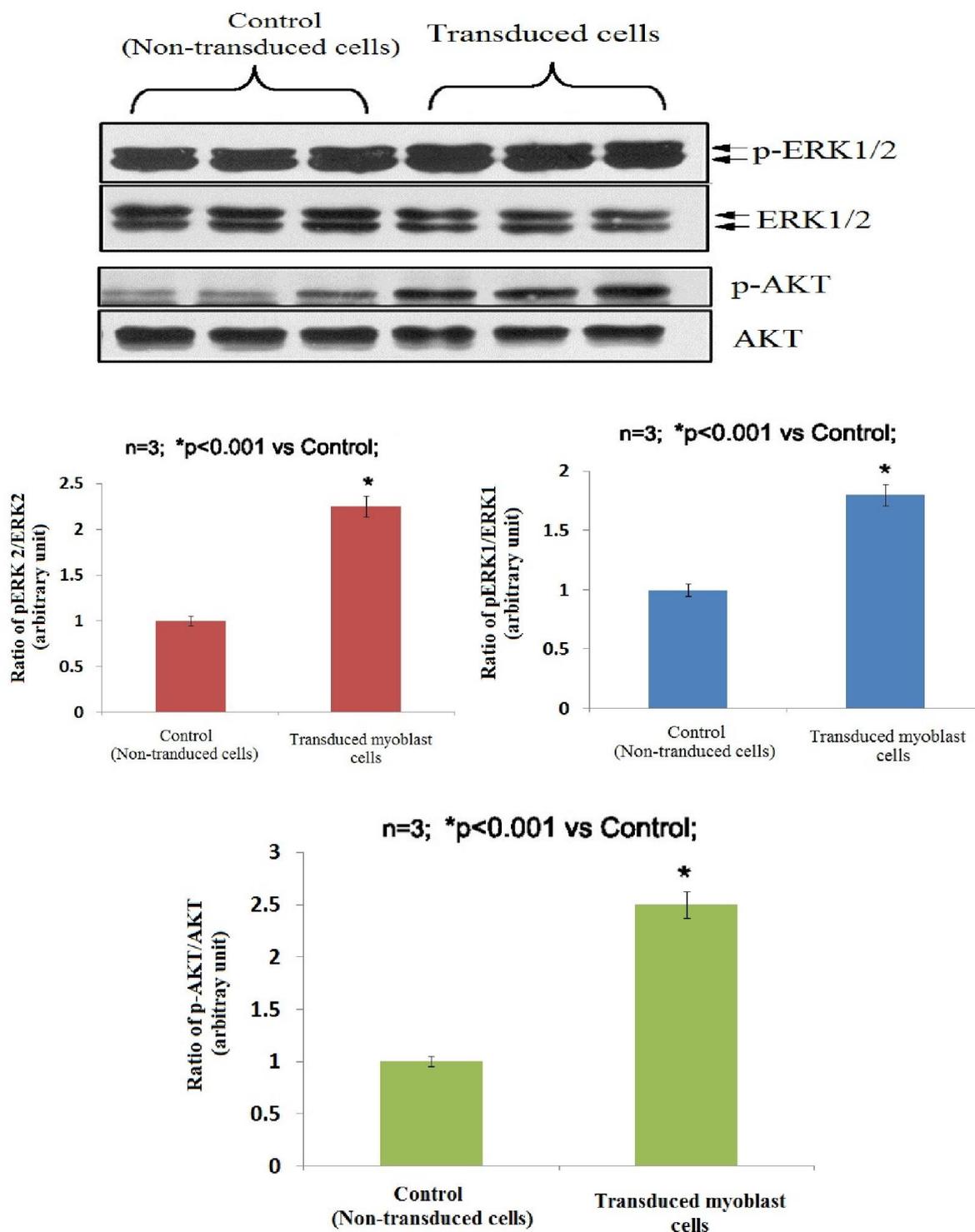
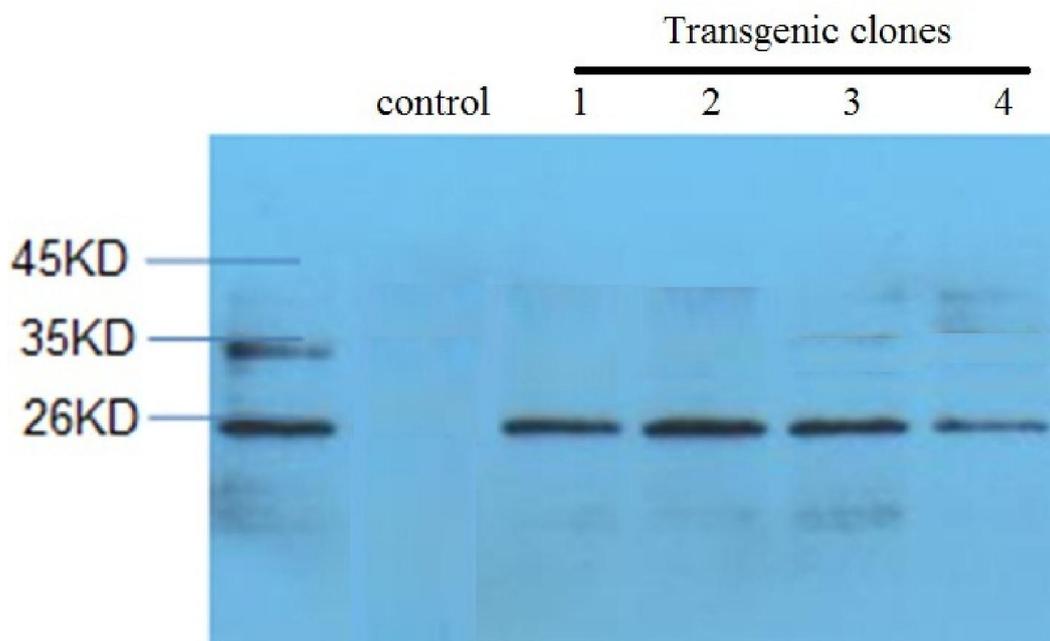
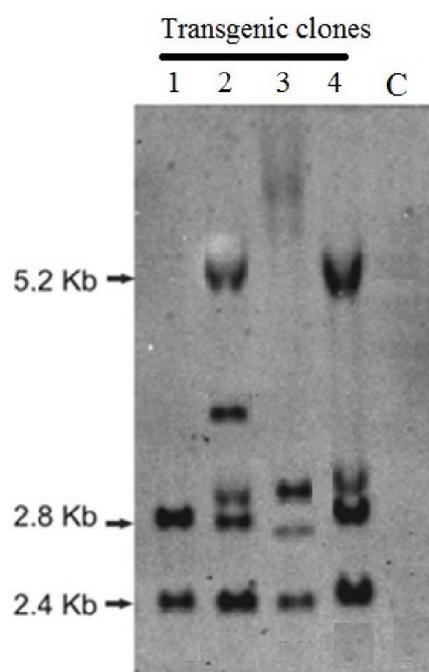


Figure (3) Effects of follistatin over-expression on the phosphorylation of ERK1/2 and Akt protein. Cell lysates were analyzed by immunoblotting using an antibody that was phospho-specific or specific for total ERK1/2 (upper panel) or Akt (lower panel), respectively.



A



B

Fig (4)A, Western blot analysis was performed to detect GFP protein expression in four transgenic myoblast clones. Anti GFP antibodies specifically recognized a 26-kDa band for GFP protein in the four transgenic myoblast clones. Non-transgenic myoblast cells were used as a control. B, Southern blot analysis of genomic DNA extracted from four transgenic clones (1, 2, 3 and 4) and non-transgenic myoblast (control).

Table I. Fusion percentage average in myotubes in ovine myoblast cultures (mean  $\pm$  S.E.M)\*

Percentage of nuclei in myotubes in ten different experiments (mean $\pm$ S.E.M.)*											
Conditions	1	2	3	4	5	6	7	8	9	10	Mean
control	25.54 $\pm$ 3.71	35.81 $\pm$ 1.09	26.21 $\pm$ 3.50	32.81 $\pm$ 2.76	35.64 $\pm$ 3.34	32.37 $\pm$ 3.52	27.63 $\pm$ 2.82	35.94 $\pm$ 3.59	31.73 $\pm$ 3.08	36.90 $\pm$ 2.39	32.05 $\pm$ 2.98
AAV- CFS-Flag	57.41 $\pm$ 4.91	50.82 $\pm$ 2.78	56.77 $\pm$ 4.30	43.61 $\pm$ 3.71	52.35 $\pm$ 2.33	53.11 $\pm$ 4.34	43.21 $\pm$ 4.31	43.71 $\pm$ 2.20	62.30 $\pm$ 2.10	51.60 $\pm$ 2.50	47.11 $\pm$ 3.34

\*The percentages are the mean  $\pm$  S.E.M. for nine counts in each well.