SUPPRESSION OF CASPASE-9 INCREASES PROLIFERATION OF HANWOO CATTLE SATELLITE CELLS

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Abstract- Catalysis of apoptotic caspases lead to wholesale destruction of cellular proteins which is a major cause of cellular death. Caspase-9 has been reported as the key regulator of apoptosis however its role in skeletal muscle cell development and the molecular involvements during proliferation still remains unknown. The current study aimed to present the key role of caspase-9 using RNA interference mediated silencing and to observe the mRNA expression level of apoptotic caspase genes as well as the genome during satellite cell growth. Three small interference RNA sequences (siRNAs) targeting caspase-9 gene was designed and ligated into pSilencer plasmid vector to construct shRNA expression constructs. Satellite cells were transfected with the constructs for 48 h. Results indicated that all three siRNAs could silence the caspase-9 mRNA expression significantly. Suppression of caspase-9 led to the increase in the expression of caspase-3, cell proliferation, adhesion and division-regulating genes whereas the reduction in the expression of cell death program and stress response-regulating genes. Otherwise, the suppression of caspase-9 did not cause a cell dead whereas it improved cell proliferation. From the obtained results it is suggested that besides the apoptotic regulation, the caspase-9 may also play some role in the skeletal muscle satellite cell proliferation.

Key words-Knockdown, caspase-9, effector caspases, genome, RNA interference, skeletal satellite cell.

I. INTRODUCTION

The caspase-9 has been found as the key regulator of apoptosis; the caspase-9 involves the release of cytochrome c from mitochondria, which then binds and activates apoptotic protease-activating factor-1 (Apaf-1) which then activates procaspase-9. The procaspase-9 is recruited to the apoptosome, is activated and undergoes auto-catalytic cleavage before it dissociates from the apoptosome and becomes inactive. Active caspase-9 at the apoptosome proteolytically activates procaspase-3. Subsequently, caspase-3 and other executioner caspases (e.g., caspase-7) execute apoptotic cell death [1, 2]. More to the point, in recent years RNA interference (RNAi) mediated by small interfering RNA (siRNA) has been widely used in gene function studies [3, 4]. The RNAi method usually utilizes short hairpin RNA (shRNA) expressed from plasmid vectors, and the shRNA-expressing construct is integrated into the genome, it becomes a sustainable source of siRNA and produces sustained RNAi effects. To the best of our knowledge; no scientific information regarding the role of caspase-9 in cell proliferation and its effect on the expression of genomic system in bovine skeletal muscle satellite cells during proliferation is available. Thus, the current work was designed to study the key role of caspae-9 in cell proliferation and the expression of genomic system using RNAi mediated silencing of caspae-9 during cell growth.

II. MATERIALS AND METHODS

2.1. Cell preparation and culture

The satellite cells were isolated from 24-month old Korean native cattle (Hanwoo) following Dodson et al. (1987) method

2.2. Designing of siRNA and construction of plasmid vectors

Three siRNA sequences against caspase-9 gene were designed and synthesized. The three target shRNA sequences for constructing pSilencer vector against caspase-9 were named as shRNA1 top: (5′-gatccgtgagcgaggtgatgaagctttcaagagaagcttcatcacctcgctcatttttttggaaa-3′) and shRNA1 bottom: (5′-agcttttccaaaaaatgagcgaggtgatgaagcttctcttgaaagcttcatcacctcgctcacg-3′); shRNA2 top: (5′-gatccggacgagaactacgacctgttcaagagacaggtcgtagttctcgtccttttttggaaa-3′) and shRNA2 bottom: (5′-agcttttccaaaaaaggacgagaactacgacctgtctcttgaacaggtcgtagttctcgtccg-3′); shRNA3 top: (5′-gatccggagagcttcgagaactacttcaagagagtagttctcgaagctctccttttttgaaa-3′) and shRNA3 bottom: (5′-agcttttccaaaaaaggagagcttcgagaactactctcttgaagtagttctcgaagctctccg-3′). These oligo sequences were annealed and the resulting annealed shRNAs were ligated into pSilencer hygro vectors by T4 DNA ligase between the BamH I and Hind III restriction sites according to the manufacturer's instructions. The shRNA expression construct was transformed into *Escherichia coli* GC^{TM} competent cells following the manufacturer's protocol.

2.3. Transfection of shRNA expression constructs

The isolated satellite cells were grown in growth medium in a humidified incubator at 37 ^{o}C with 5% CO_2. Cells were transfected with either shRNA expression constructs or pSilencer hygro vector negative control for control using Lipofectamine 2000^{TM} reagent according to the manufacturer's instructions.

2.4. Confocal scaning laser microscope (CSLM)

The transfected cells were incubated for 48 hr and then stained with contents of live/dead cytotoxicity kit following our previously established method [5]. Finally the stained cells were observed under the fluorescence microscope.

2.5. Cell proliferation assay

To assay the impact of caspase-9 suppression on satellite cell proliferation, the cell proliferation was determined by using CCK-8 colorimetric assay according to the manufacturer's instruction.

2.6. Quantitative real time polymerase chain reaction (qRT-PCR)

The primers and conditions for the RT-PCR of genes were followed our standardized [6].

2.7. *Microarray analysis*

Gene expression profile followed the transfection was assayed using the bovine (V2) gene expression 4 x 44K microarray (Agilent Technologies) according to the manufacturer's instruction.

2.8. Statistic analysis

ANOVA test was applied to test the significance of difference between the cells transfected by three different shRNA expression constructs with control cells. All mean values were compared using the Duncan's multiple-range test at the 5% level of significance (SAS Institute, Cary, NC, 2007).

III. RESULTS AND DISSCUSION

In the present study, three shRNA expression constructs were constructed. Following optimizing the transfection conditions, qRT-PCR were used to assess the inhibition level of caspase-9 expression achieved by all used siRNA sequences. Our results show that the relative mRNA expression level of caspase-9 gene in the cells transfected by shRNA1, shRNA2 and shRNA3 constructs were reduced by 46.78%, 73.01% and 34.48%, respectively when compared with the control cells (Fig. 1). Several workers have also reported that the efficient silencing of specific genes by RNAi approach using plasmid vectors to transfect into the fibroblast cells [7].

Three separate shRNA expression constructs were screened for their ability to silence caspase-9 expression in satellite cells.

Following optimizing the transfection conditions, the CCK-8 assay was used to assay the effect of caspase-9 knockdown on the cell proliferation. Fig. 2a shows CCK-8 data of three different shRNA expression constructs at 48 h post-transfection. The obtained data showed a slight increase in the cell proliferation following the shRNAs transfection compared with control cells but no differences ($P > 0.05$). It is probably that the suppression of caspase-9 may reduce the impacts from the environmental factors around such as stress due to the transfection reagents, and thus activates the cell division and proliferation. Further, the cell morphology after transfections was also studied using CSLM. Fig. 2 shows the representative CSLM images of satellite cells following 48 h transfection. Once can observe that no dead cells were observed in the shRNA2-transfected cells as well as the control cells (Fig. 2d and 2e).

In order to understand the involvements of caspase-9 in the regulation of other gene expressions we further studied the expressions of genome of the shRNA2-transfected cells following 48 h tranfection by using microarray analysis. Interestingly, we observed that 13,781 genes were up-regulated, whereas 14,016 genes were down-regulated after the transfection. On the other hand, we compared the gene expression profile in the shRNA2-transfected cells and the control cells. In the scatter plot shown in Fig. 3, the position of each gene on the plot is determined by its expression level in both shRNA2-transfected cells (*x*-axis) and control cells (*y*-axis). Genes with identical or similar expression are depicted by a black line whereas the gene clusters around the red line depicts the ≥ 2 fold up-regulation. The green solid line demonstrates the ≤ 2 fold down-regulation. The red spots at the top of red line indicate ≥ 3 fold up-regulation whereas the blue spots at the bottom of green line illustrate ≤ 3 fold down-regulation respectively. We identified 538 genes (1.72% of 31,234 genes examined) whose expression level in shRNA2-transfected cells was ≥ 2 fold higher than those of control cells, and 327 genes (1.04% of 31,234 genes examined) whole expression level was ≤ 2 fold lower than those in control cells. By comparing the shRNA2-transfected cells with the control cells, we identified 41 genes which were significantly down-regulated (< 5 folds) after transfected by the shRNA2. These down-regulated genes were further grouped according to the biologically functional categories including; inhibition of cellular biosynthetic process (10), regulation of programmed cell death (17 genes), stress response (6), inhibition of cell proliferation (4 genes) and negative regulation of multicellular organismal process (4 genes). On the other hand, we identified 60 genes that were significantly up-regulated (> 5 folds) after transfected by the shRNA2. These up-regulated genes were also grouped according to the biologically functional categories including; regulation of cell proliferation (19 genes), inhibition of apoptosis (14 genes), cell adhesion (23genes) and regulation of cell division (4 genes).

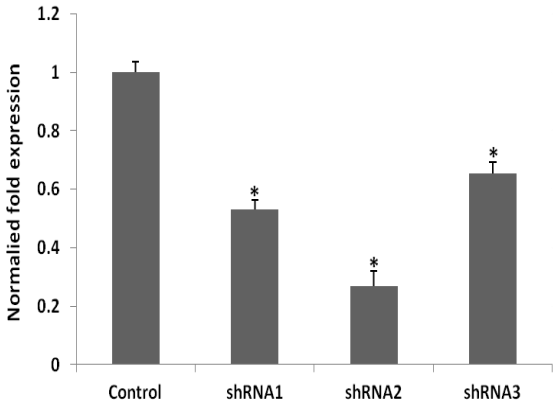

Figure 1 Knockdown of caspase-9 in satellite cells analyzed at mRNA level. The mRNA expression was assayed by RT-PCR and following 48 h transfection. RT-PCR with specific primers confirmed that caspae-9 was silenced at the mRNA level. Value expressed as mean ± standard error of mean (SEM), calculated from three independent experiments. Gene expression was

normalized to GAPDH expression and denoted as a gene/GAPDH ratio, *P <0.05.

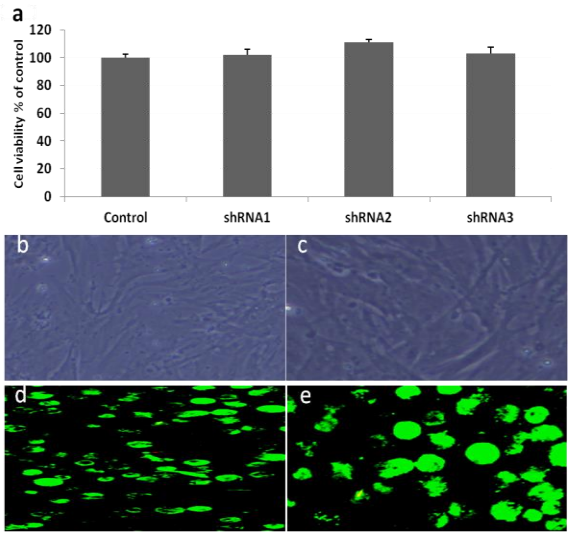

Figure 2 Effect of caspase-9 suppression on satellite cell proliferation. (A) The proliferation of cells following transfection as indicated was assayed by the CCK-8 assay. Data represent the mean ± standard rror of mean (SEM) of triplicates. Representative images of transfected cells which were taken by electronic microscope at 40 x magnification, (B) represents the control cells and (C) represents the shRNA2-transfected cells at 48 h; (D) represents the confocal scanning laser microscope (CSLM) image of control cells; (E) represents the CSLM image of shRNA2- transfected cells at 48 h. The cells were stained by DiOC18 (3)/PI. Dead cells are labeled by PI and have red nuclei. Live cells are labeled by DiOC18 (3) and have green nuclei.

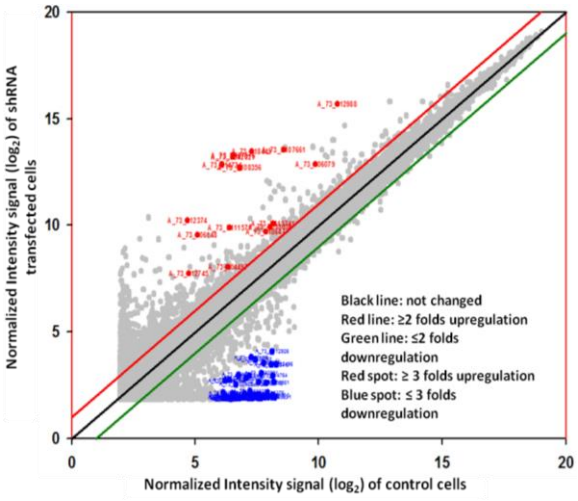

Figure 3 Expression profiling of genes in shRNAs-transfected cells and control cells. The gene expression profiles of transfected cells or control were assessed using oligonucleotide microarray analysis. A total of 31,324 genes present on the bovine gene expression microarray were plotted using a scatter plot. The position of each gene on the plot is determined by its expression levels in control (*x*-axis) and shRNAs-transfected cells (*y*-axis).

IV.CONCLUSION

The suppression of caspase-9 led to the increase in the expression of cell proliferation, adhesion and division-regulating genes whereas the reduction in the expression of cell death program and stress response-regulating genes, suggesting that the caspase-9 play an important role in the cattle skeletal satellite cell proliferation.

ACKNOWKLEGMENT

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