μ-CALPAIN GENE KNOCKDOWN OF MUSCLE SATELLITE CELLS USING PSILENCER VECTOR

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Abstract – This study was designed to find the key role of μ-calpain and caspases using RNA interference mediated silencing of μ-calpain and caspase 9 during satellite cell proliferation. For this experiment, four separate siRNA sequences were screened for their ability to inhibit μ-calpain gene expression. Following optimizing the transfection conditions for cell number, volume of transfection agent used and the appropriate concentration of each siRNA, quantitative real-time PCR were used to assess the level of inhibition of gene expression achieved by all siRNA sequences used. CAPN1, caspase 3, caspase 7, caspase 9, Hsp27, Hsp70 and Hsp90 mRNA expression were quantified in CAPN1-siRNA treated cells by real-time PCR analysis. As a result, knock-down of μ-calpain lead to the reduction in caspase 3 and caspase 7 gene expression, suggesting that there were a cross-talk between μ-calpain and the caspase enzyme systems. In addition, targeted suppression of caspase 9 gene expression reduced caspase 7 activation and we hypothesized that apoptosis take place through an intrinsic pathway during satellite cell proliferation.

Key Words – μ -calpain, satellite cell, Hanwoo.

I. INTRODUCTION

Muscle tissue possesses the post-natal growth and intrinsic regenerative capacity (1). Comprehensive understanding of the satellite cells involvement in postnatal myogenesis, skeletal muscle hypertrophy and myofiber regeneration are noteworthy issue for fundamental agricultural reasons. Calpains are intracellular nonlysosomal Ca^{2+}-regulated cysteine proteases and it mediate regulatory cleavages of specific substrates in various cellular processes such as signal transduction, cell proliferation and differentiation, apoptosis and necrosis in mammals (2). Muscle tissue expresses mainly three distinct calpains: the ubiquitous calpains 1 and 2 (also called μ- and m-) which are the best-characterized calpains and calpain 3 (also called p94) which is highly expressed in this tissue. However, since conventional inhibitors used for the studies of the functions of these enzymes lack specificity, the individual physiological function and biochemical mechanism of these three isoforms, especially μ-calpain, are not clear (3). In contrast, RNA interference (RNAi) has a great potential to distinguish the functions of each member in a closely related gene family or to selectively target a mutant gene, especially to study the functions of a particular isoform. Recently numerous research revealed that the potential role of calpains involving apoptosis is indicated by a increasing list of calpains substrates such as p53, PARP, Bax, AIF and several cytoskeletal proteins (2). Although calpains are known to contribute to apoptosis, further studies are still needed to precisely elucidate the role of calpains in apoptosis. Recently a cross talk between the calpain and the caspase systems has been reported (4). Caspases is another family of proteases which are involved in programmed cell death. The caspases that appear to play a role in these processes such as initiator caspases (caspase-2, -8, and-9) and effector caspases (caspase-3, -6, -7, and -14) (5). The role of these caspases in muscle cell development or differentiation process in Hanwoo cattle is very limited. Earlier studies from our reported that elevation of caspase 9 expression during satellite cell proliferation and differen-tiation. In continuance to our earlier investigations, the present work was designed to study the key role of μ-calpain and caspases using RNA interference mediated silencing of μ-calpain and caspase 9 during satellite cell proliferation.

II. MATERIALS AND METHODS

All chemicals and laboratory wares were purchased from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA) and Falcon Lab ware (Becton-Dickinson, Franklin Lakes, Nj, USA), respectively. Satellite cells were isolated from 30-month-old Korean Hanwoo cattle according to the method of Dodson et al. (6). siRNA-mediated μ-calpain gene silencing was carried out according to method of Honda et al (7). $p^{silencer}$ 2.1 hygro vector is used for this study and primer sequences used is listed in table 1.

Statistical analysis

All the values are expressed as means ± SEM. Statistical analysis was performed using SPSS version 16.0 (Statistical Package). Student's t-test was performed to determine the differences between control and treatments. $p \leq 0.05$ was considered to be significant.

III. RESULTS AND DISCUSSION

In present study, there are four separate siRNA sequences were screened for their ability to inhibit μ-calpain gene expression. Following optimizing the transfection conditions for cell number, volume of transfection agent used and the appropriate concentration of each siRNA, quantitative real-time PCR were used to assess the level of inhibition of gene expression achieved by all siRNA sequences used. CAPN1, caspase 3, caspase 7, caspase 9, Hsp27, Hsp70 and Hsp90 mRNA expression were quantified in CAPN1-siRNA treated cells by real-time PCR analysis. CAPN1-siRNA3 and CAPN1-siRNA4 mRNA expressions significantly reduced compared to control (Figure 1). Caspase 3 expression not significantly changed in CAPN1-siRNA transfected satellite cells (Figure 2). Caspase 7 expression significantly reduced in CAPN1-siRNA2, CAPN1-siRNA3 and CAPN1-siRNA4 transfected satellite cells (Figure 3). Caspase 9 expression significantly increased in CAPN1-siRNA2, CAPN1-siRNA3 and CAPN1-siRNA4 transfected satellite cells (Figure 4). Hsp27 expression significantly increased in CAPN1-siRNA2, CAPN1-siRNA3 and CAPN1-siRNA4 transfected satellite cells (Figure 5). Hsp70 expression significantly increased in CAPN1-siRNA3 and CAPN1-siRNA4 transfected satellite cells (Figure 6). Hsp90 expression significantly increased in CAPN1-siRNA2, CAPN1-siRNA3 and CAPN1-siRNA4 transfected satellite cells (Figure 7).

In this study, primary satellite cell cultures are derived directly from adult Korean Hanwoo *longissimus dorsi* muscle. The *in vitro* properties exhibited by primary cultures of satellite cell more closely reflect their *in vivo* properties than those exhibited by transformed cell lines (8). Furthmore, Primary cell culutres, transformed cell lines cutures and isolated myofiber cultures have all been well used to undertake general aspects of satellite cell physilogy and satellite cell regulation. Even though, there are remains hotly debated on the use of these in vitro systems, both critics and proponents have tended to agree that the use of in vitro systems for determining the developmental biology of satellite cells has resulted in considerable, and useful, data (9). Numerous papers have been published involving the animal as a major experimental unit that were to develop species-specific satellite cell. However, all of these satellite cell researchers worry about few contaminating cells such as fibroblasts, which co-isolated with satellite cells during cell isolation regimens because these contaminating cells protentially could overrun cultures and provide biased measures of cell activity *in vitro* (9). In this study, to decrease the presence of non-myogenic cells in primary cultures, the cell suspensions were loaded onto a magnetic cell sorting system, AutoMACS (Milteny Biotec, Germany) to isolate the satellite cells which strengthened insure that culture systems were controlled and interpretable. Herein, caspase 9 acts as an initiator caspase and caspase 7 acts as an effector caspase which are possibly involved in satellite cell proliferation.

However, in present study knock-down of caspase 9 expression in satellite cells led to an increase in caspase 3 expressions, probably satellite cells were stressed from exposure to high concentration (30 nM) transfection agent/siRNA complex and different pathways of apoptosis happened. There is still a possibility that other initiator/effector caspases and proteinases carried out the role of caspase-3 in proliferation muscle satellite cell. Taken together, herein we hypothesized that apoptosis take place via a mitochondrial pathways during cattle muscle satellite cell proliferation. Apoptosis is a genetically programmed form of cell death that can be triggered through death

receptors such as the TNF-receptor or via mitochondrial pathways (10)). The intrinsic death signaling pathway is induced by the release of cytochrome c from mitochondria. Cytochrome c, apoptosis-activating factor-1 (APAF-1) and caspase 9 then form a complex called the apoptosome which results in the activation of caspases 7 as the down-stream effector caspases. Our findings indicates that knock-down of µ-calpain lead to the reduction in caspase 3 and caspase 7 gene expression, suggesting that there were a cross-talk between µ-calpain and the caspase enzyme systems. In addition, trageted suppression of caspase 9 gene expression reduced caspase 7 activation and we hypothesized that apoptosis take place through an intrinsic pathway during satellite cell proliferation.

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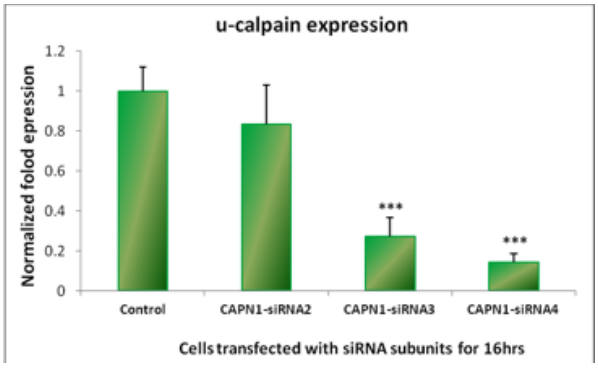

Figure 1. µ-calpain expression in CAPN1siRNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and µ-calpain expression was normalized to GAPDH reference gene expression.***p<0.001

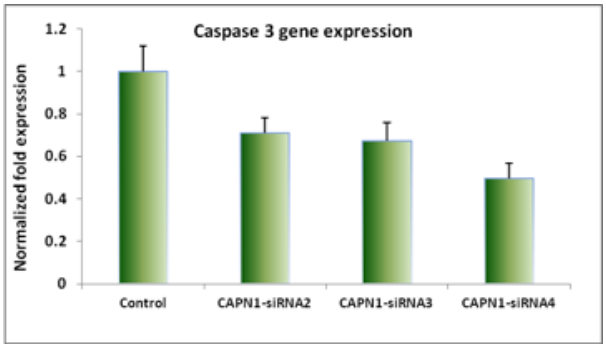

Figure 2. Caspase 3 expression in CAPN1si RNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and Caspase 3 expression was normalized to GAPDH reference gene expression.

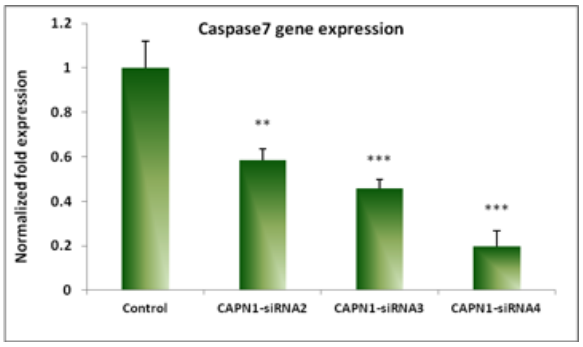

Figure 3. Caspase 7 expression in CAPN1siRNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and Caspase 7 expression was normalized to GAPDH reference gene expression. **p<0.01, ***p<0.001

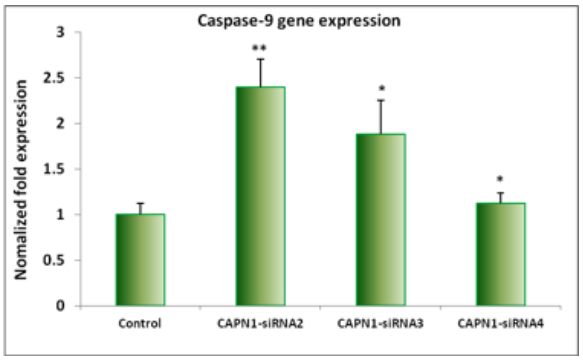

Figure 4. Caspase 9 expression in CAPN1si RNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and Caspase 9 expression was normalized to GAPDH reference gene expression. *p<0.05, **p<0.01

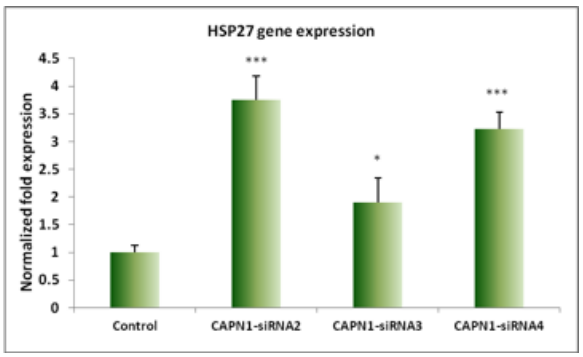

Figure 5. HSP27 expression in CAPN1siRNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and HSP27 expression was normalized to GAPDH reference gene expression. *p<0.05, **p<0.01, ***p<0.001

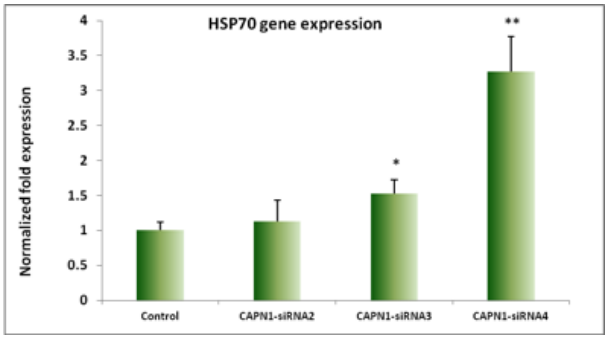

Figure 6. HSP70 expression in CAPN1siRNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and HSP70 expression was normalized to GAPDH reference gene expression. *p<0.05, **p<0.01

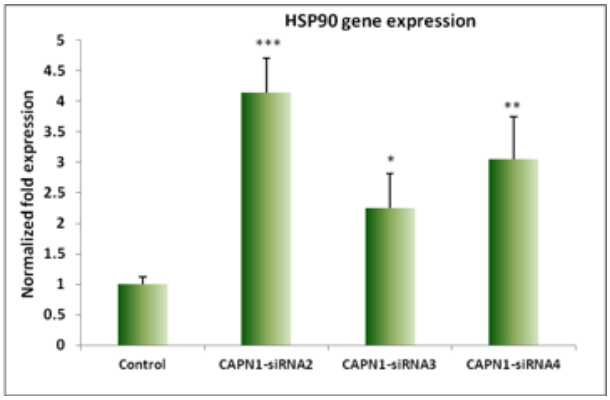

Figure 7. HSP790 expression in CAPN1siRNAs-transfected muscle satellite cells. The satellite cells were trasnfected with µ- calpain siRNA subunits 2, 3 & 4 (CAPN1siRNAs) for 16hrs and HSP90 expression was normalized to GAPDH reference gene expression. *p<0.05, **p<0.01, ***p<0.001