

Glibenclamide elicits endoplasmic reticulum stress and myokine expression in the L6 skeletal muscle cells

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Abstract—Although glibenclamide is an oral hypoglycemic agent used in type 2 diabetes, skeletal muscle wasting has been reported as a side effect. To understand how to reduce this side effect, we determined whether glibenclamide induces endoplasmic reticulum (ER) stress in skeletal muscle cells and which myokine expression changes at this time. The ER chaperone genes do not show a significant change by glibenclamide, but the ER stress sensor genes are upexpressed approximately twice, and those downstream [ATF6 (activating transcription factor 6) fragmentation, eIF2 α (eukaryotic initiation factor-2 α) phosphorylation, and XBP1 (Xbox DNA-binding protein) mRNA splicing] are activated. Additionally, the myokine gene expression was up- or downregulated by glibenclamide. These results will serve as useful data for overcoming the side effects of sarcopenia caused by glibenclamide.

Keywords—endoplasmic reticulum (ER) stress, glibenclamide, L6 skeletal muscle cells, myokines

I. INTRODUCTION

GLIBENCLAMIDE, also known as glyburide, was first synthesized in 1969 and has been used in aged patients affected by impaired glucose-induced insulin release in the United States since 1984. Presently, glibenclamide is commonly used as an antidiabetic agent in managing type 2 diabetes [1]. It inhibits K_{ATP} in pancreatic beta cells, thereby activating the voltage-sensitive calcium channel, which increases the

intracellular calcium concentration and accelerates insulin secretion [2]. It has been reported that physiological side effects of glibenclamide include nausea, heartburn, angioedema, and hypoglycemia [3]. Several other effects under biomedical research have already been reported, such as apoptosis induction, inhibition of *extracellular matrix* formation, inhibition of cell growth, and increase in twitch and tetanus tension by glibenclamide [4-7]. Although another beneficial function of glibenclamide is reported to reduce edema-hemorrhage in traumatic brain injury and apoptosis induction in cancer cells, the most interesting side effect is skeletal muscle wasting associated with type 2 diabetes [8]. As many aged people have already been exposed to sarcopenia and diabetes, glibenclamide is used as an oral drug for treating type 2 diabetes. Some *in vitro* studies reported that muscle atrophy associated with type 2 diabetes was a side effect of glibenclamide; it was also demonstrated that K_{ATP} mainly acts as a molecular sensor in skeletal muscle atrophy [9]. The International Working Group on Sarcopenia defined sarcopenia as “age-associated loss of skeletal muscle mass and function,” in 2011 [10] WMC and the European Working Group on Sarcopenia in Older People declared it as “a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes, such as physical disability, poor quality of life, and death” in 2019 [11].

The endoplasmic reticulum (ER) of eukaryotic cells is a critical site for posttranslational modification, including protein folding and assembly. ER lumen has many ER chaperones

[binding immunoglobulin protein (BiP), calnexin, protein disulfide isomerase (PDI), and ER protein 29 (ERp29)] responsible for various functions. Furthermore, three types of ER stress sensors [inositol requiring enzyme 1 (IRE1), PKR like ER kinase (PERK), and activating transcription factor 6 (ATF6)] are activated [12]. ER stress in mammalian cells is triggered by the dissociation of BiP due to stress transducers, such as PERK, ATF6, and IRE1. As ER stress signal and other intercellular signals are closely correlated, it is frequently checked up during the initial stage of developing new drugs, in addition to drug efficacy and side effect verification. In this study, we tested whether glibenclamide induces ER stress signal in L6 skeletal muscle cells. By understanding the ER stress signal using glibenclamide in muscle cells, we expect to provide a better understanding of how to reduce the side effects of sarcopenia induction by glibenclamide.

II. MATERIALS AND METHODS

L6 rat myoblast-derived cells were maintained at 37°C with 5% CO₂ in collagen coated flasks containing DMEM supplemented with 10% FBS and 100 µg/ml penicillin-streptomycin antibiotic [13]. When the cells showed >90% confluence, they were maintained with no changes in culture medium for 2-3 days more to induce myotube formation. Glibenclamide (C₂₃H₂₈ClN₃O₅S; 494.004 g/mol) purified to ≥99% (HPLC) was purchased from Sigma Aldrich. Total RNA was extracted from L6 cells using the SV Total RNA Isolation System (Promega, Madison, WI, USA) and quantified using the NanoDrop Lite UV-spectrophotometer (Thermo Scientific, Waltham, MA, USA).

The mRNA in the samples was reverse-transcribed using the SuperscriptIII™ First Strand Kit (Invitrogen Carlsbad, CA, USA). RT PCR conditions included 30 cycles as follows: 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min (10 min in the final cycle) using primers with Taq DNA polymerase. The RT-PCR bands were quantitatively analyzed using the ImageJ program of NIH. The RT PCR primers supplied by Bioneer Co. (Daejeon, Korea) and used in this study are listed in Table 1 (BiP; binding immunoglobulin protein, Canx; calnexin, PDI; protein disulfide isomerase, ERp29; ER protein 29, PERK; protein kinase RNA-like ER kinase, ATF6; activating transcription factor 6, IRE1; inositol requiring protein 1, XBP1; X-box binding protein-1, LC3a; autophagy marker light chain 3a, Beclin, Bax; BCL2 associated X, Bak, BCL-2 killer, Bcl-2; B-cell lymphoma 2, Bid; BH3-interacting domain death agonist, MyoD1; myogenic differentiation 1, MyoG; myogenin, Vfgfa; vascular endothelial growth factor a, BMP7; bone morphogenetic protein 7, IL-6; interleukin-6, IL15 interleukin-15, Apln; apelin, Sesn1; sestrin 1, SDF1; stromal cell-derived factor 1, SPARC; secreted protein acidic and rich in cysteine, Visfatin, Musclin, Dcn; decorin, PPIA; peptidylprolyl isomerase A). The size difference between spliced and unspliced XBP1 mRNA was measured. Total RNA was reverse-transcribed, and double strand cDNA was synthesized by PCR using specific sense and

anti-sense primers described in Table 1. The amplified cDNA was treated with PstI, and the resulting products were analyzed by electrophoresis in a 2.5% agarose gel. All undescribed chemicals were purchased from Sigma Aldrich.

Western blotting was performed briefly with modifications and L6 cells were lysed using RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride). The total protein concentration was estimated using Pierce BCA protein assay Kits (Thermo Fisher Scientific, Cleveland, OH, USA). Proteins were separated using SDS-PAGE and transferred to a nitrocellulose membrane. The resulting membrane was incubated with primary antibodies overnight at 4°C, followed by incubation with secondary antibodies. It was developed using an enhanced chemiluminescence Western blotting detection system Kit (Amersham, Sweden) according to the manufacturer's instructions. Anti-ATF6, anti-eIF2 α , anti-eIF2 α -P, and goat anti-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

III. RESULTS AND DISCUSSION

We tested the effect of glibenclamide in inducing the expression of ER chaperones (BiP, Calnexin, PDI, and ERp29) and ER stress sensors (PERK, ATF6, and IRE1). BiP is an ER chaperone located in the ER lumen, which binds newly-synthesized proteins during translation. However, it is bound to misfolded proteins and ER stress sensors under ER stress conditions. Calnexin plays a vital role in the proofreading of misfolded glycoproteins in the ER lumen. PDI is also located in the ER lumen, which catalyzes the formation and breakage of disulfide bonds between cysteine residues within folding proteins. ERp29 is highly homologous with PDI and is involved in processing secretory proteins (12)

The expression of BiP and ERp29 was approximately 2.5 and 4 times higher than ER stress-inducible tunicamycin, respectively, and only approximately 1.5 times higher than PDI and ERp29 by glibenclamide. The result of Fig. 1A suggests that glibenclamide does not induce remarkable expression of ER chaperones. PERK is a type I transmembrane protein kinase that inhibits the overall protein translational process by phosphorylation of eIF2 by ER stress. ATF6 is an ER membrane-type II protein that produces ATF6c transcription factor cleaved by S1P and S2P upon ER stress. IRE1 is autophosphorylated by ER stress to induce XBP1 mRNA splicing. The expression of three ER stress sensors by glibenclamide was almost the same as that by tunicamycin (Fig. 1B result). The result of Fig. 1B suggests that glibenclamide regulates the expression of ER stress sensors at almost the same level as tunicamycin.

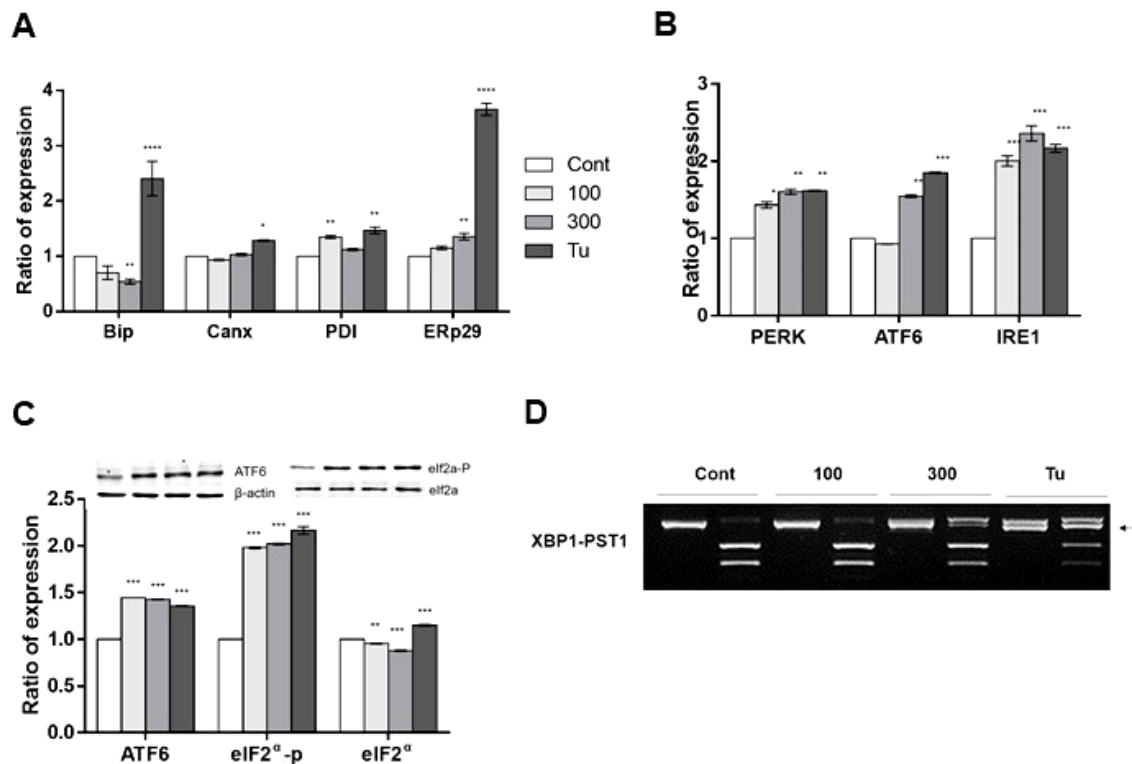


Fig. 1: The effect of glibenclamide on ER stress. (A and B) L6 cells were treated with glibenclamide (100 and 300 µg/ml) and tunicamycin (25 ng/mL) for 24 h. Gene expression of both ER chaperones (BiP, Canx, PDI, and ERp29) and ER stress sensors (PERK, ATF6, and IRE1) was demonstrated by RT-PCR. Tu, tunicamycin; Canx, Calnexin. (C) Western blotting was performed using an anti-ATF6 monoclonal antibody, anti-eIF2- α antibody, and eIF2- α -P antibody against cells treated with different doses of glibenclamide (100 and 300 µg/ml) for 24 h. (D) RT-PCR was performed for the XBP1 mRNAs. PstI further digested the resulting PCR products to reveal a lost restriction site following XBP1 splicing under ER stress. Digested XBP1 cDNA products were revealed on a 2% agarose gel. Unspliced XBP1 mRNA is indicated only by an arrow. Statistical significance between multiple groups: one-way analysis of variance test. Analysis was performed using GraphPad Prism v.6 software (GraphPad Software Inc.). Mean \pm SEM. n = 6. *p < 0.05 **p < 0.005 ***p < 0.001 ****p < 0.0001.

Three ER stress sensors regulate the expression of various genes related to protein homeostasis by activating specific signaling pathways and downstream factors. ATF6 is cleaved at the cytoplasmic N-terminus, a transcription factor of ATF6c with a bZIP domain that increases the transcription of chaperone molecules and ER-associated degradation-related genes. eIF2- α phosphorylation inhibits the activity of eIF2B, which regulates GDP-GTP exchange and interferes with the formation of complexes required for protein translation, thereby inhibiting overall protein biosynthesis. IRE1 activates the endonuclease domains, which cleave XBP mRNA, generating an activated form of the XBP1 mRNA for XBP1 protein. Next, we tested the downstream of three ER stress sensor signals, such as ATF6 fragmentation, eIF2- α phosphorylation, and XBP1 mRNA splicing [14]. The ATF6c generated by fragmenting ATF6 by glibenclamide was about 1.5 times higher than its control, and eIF2 phosphorylation was about twice as high as the control (Fig. 1C). As observed regarding the ER stress-inducible drug tunicamycin, unspliced XBP1 mRNA by glibenclamide (300 µg/ml) treatment is shown by an arrow (Fig. 1D). Although ER chaperone expression was not induced by

glibenclamide (Fig. 1A result), glibenclamide induces the expression of ER stress sensors and activates those downstream (Fig. 1B-D).

Following, we observed that glibenclamide induces gene regulation associated with autophagy, apoptosis, myogenesis, and myokines. Autophagy is a mechanism by which cells break down and recycle unnecessary or nonfunctional cell components for survival, closely related to cell death and disease induction [15]. Apoptosis is programmed cell death observed in multicellular organisms, and cells die due to cell shape and internal biochemical changes [16]. Myogenesis is the differentiation from somatic stem cells to muscle progenitor cells and myofibers in adults [17]. Skeletal muscle is an important tissue that maintains the function of our body, but recent studies have reported that secreting bioactive substances similar to various hormones secreted by endocrine organs. They are called myokines that is a compound word of myocyte, which means muscle cells, and cytokine, a kind of small hormone, which means cytokine secreted from the skeletal muscle [18, 19].

Both LC and Beclin genes were investigated after the

glibenclamide treatment in L6 cells to determine whether autophagy is induced by glibenclamide. As seen in the results of

Fig. 2A, no remarkable differentiated expression was observed compared with the control. However, the expression

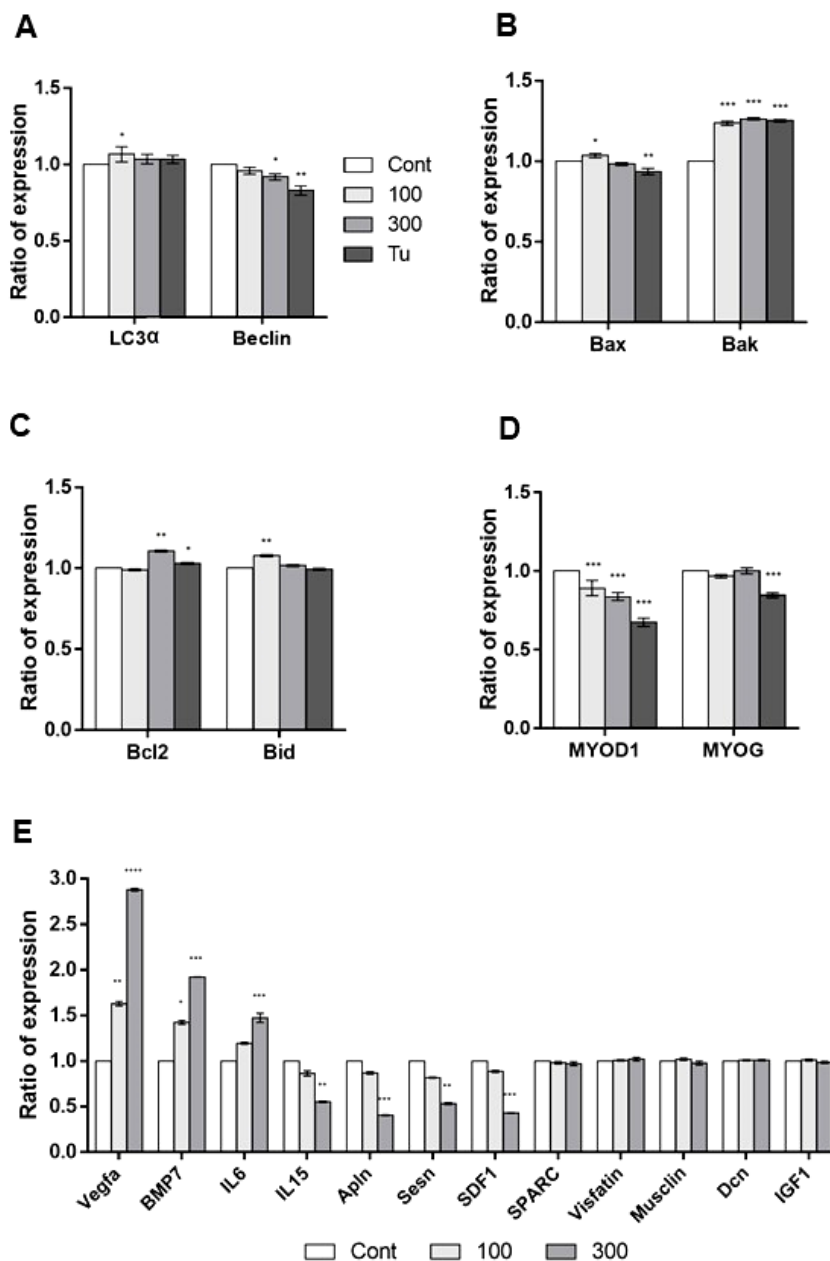


Fig. 2: The effect of glibenclamide on cell death and myokine expression. (A, B, and C) L6 cells were exposed to glibenclamide at 100 and 300 µg/ml for 24 h to confirm apoptosis and autophagy. Gene expression was measured using RT-PCR primer. LC3a, microtubule-associated protein 1A/1B-light chain 3a; Bax, Bcl2 associated X; Bak, Bcl2 Antagonist/Killer 1; Bcl2, B-cell lymphoma 2; Bid, BH3-only Bcl-2 family member. (D) The effect of glibenclamide on gene expression of myogenesis factors. MyoD1, myogenic differentiation 1; MyoG, myogenin. (E) The effect of glibenclamide on gene expression of myokines. VEGFA, vascular endothelial growth factor-a; BMP7, bone morphogenetic protein 7; IL6, interleukin 6; IL15, interleukin 15; Apln, apelin; Sesn, sestrin; SDF1, stromal cell-derived factor 1; SPARC (osteonectin), secreted protein acidic and rich in cysteine; Dcn, decorin; IGF1, insulin-like growth factor-1. Mean ± SEM. n = 6. *p < 0.05 **p < 0.005 ***p < 0.001 ****p < 0.0001.

of apoptosis-related genes by glibenclamide and apoptosis induction (Bax and Bak) and inhibition (Bcl2 and Bid) was investigated. No significant changes in expression were observed, except for a slight increase (about 1.3 times) of the Bak gene expression related to apoptosis induction (Fig. 2B and 2C). The results of Fig. 2A-C indicate that glibenclamide is not actively involved in the induction of autophagy and apoptosis; glibenclamide did not affect the degradation of the muscle cells. Moreover, the gene expression of myogenic regulatory factors of MyoD1 and MyoG was investigated to determine the effect of glibenclamide on muscle differentiation. Glibenclamide weakly suppresses the expression of the MyoD1 gene at a high concentration, but no change is observed in the case of MyoG (Fig. 2D). Finally, we tested 12 myokines to determine how glibenclamide affects myokine expression in the muscle cells (Fig. 2E). Expression patterns were divided into three: gene expression increased, descended, and unchanged. The gene expression of VEGFA was increased by about three times, BMP7 about twice, and IL6 about 1.5 times compared with the control. The gene expression reduced by half in IL15, aplin, sesn, and SDF1, and the rest showed no significant change.

In summary, although gene expression of ER chaperones (BiP, Calnexin, PDI, and ERp29) was not affected by glibenclamide, which activated the genes of ER stress sensor (PERK, ATF6, and IRE1) and those downstream, such as ATF6 fragmentation, phosphorylation of eIF2- α , and XBP1 mRNA splicing. Furthermore, gene expression related to autophagy and apoptosis was not affected by glibenclamide. However, myokines of VEGFA, BMP7, and IL6 gene expression were selectively increased by glibenclamide, whereas myokines of IL15, aplin, sesn, and SDF1 were selectively suppressed. Even if our results can fully explain sarcopenia induction by glibenclamide, they provide an opportunity to observe the behavior of related factors in muscle cells and not harmful effects. Depolarization occurs due to increased intracellular K⁺ because of KATP inhibition by glibenclamide, and a large amount of Ca⁺⁺ is introduced through the voltage-sensitive calcium channel [20-22]. In case the delicate mechanisms are resolved singularly by comprehensively considering the changes in intracellular environments caused by glibenclamide and selective expression of myokines genes, a better understanding of the solution of skeletal muscle wasting caused by glibenclamide can be expected.

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Contribution of Individual Authors to the Creation of a Scientific Article (Ghostwriting Policy)

Seung-Whan Kim, O-Yu Kwon: Conceptualization, Resources, Supervision, Project administration, Writing Original Draft.

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Table 1. The gene names, GenBank numbers, and sequence of the primers used in the RT-PCR experiments.

Gene	NCBI Reference Sequence	Primer sequence
BiP	NM_013083.2	F 5'-AGT GGT GGC CAC TAA TGG AG-3' R 5'-TCT TTT GTC AGG GGT CGT TC-3'
Canx	NM_172008.2	F 5'-GGC ATC TTC ATC CCA GTC AT-3' R 5'-CTC CTC TCT GCT CCT CAT GG-3'
PDI	NM_012998.2	F 5'-CCA AAG CTG CTG CAA AAC TG-3' R 5'-AAA GAG GAC CAC CCC ATC CT-3'
ERp29	NM_053961.2	F 5'-GGC ATC TTC ATC CCA GTC AT-3' R 5'-TCC TCT GCA GCT CTT CCT TC-3'
PERK	NM_031599.2	F 5'-GGT CTG GTT CCT TGG TTT CA-3' R 5'-TTC GCT GGC TGT GTA ACT TG-3'
ATF6	NM_001107196.1	F 5'-CTA GGC CTG GAG GCC AGG TT-3' R 5'-ACC CTG GAG TAT GCG GGT TT-3'
IRE1	NM_001191926.1	F 5'-ACC ACC AGT CCA TCG CCA TT-3' R 5'-CCA CCC TGG ACG GAA GTT TG-3'
XBP1	NM_001004210.2	F 5'-TCC TCC TGG GTA GAC CTC TG-3' R 5'-AAA CAG AGT AGC AGC TCA GA-3'
LC3a	NM_199500.2	F 5'-GCC TGT CCT GGA TAA GAC CA-3' R 5'-GTT CAC CAG CAG GAA GAA GG-3'
Beclin	NM_001034117.1	F 5'-GTG CTC CTG TGG AAT GGA AT-3' R 5'-GCT GCA CAC AGT CCA GAA AA-3'
Bax	NM_017059.2	F 5'-AGG GGC CTT TTT GTT ACA GG-3' R 5'-GAT CAG CTC GGG CAC TTT AG-3'
Bak	NM_053812	F 5'-TTA CCT CCA CCA GCA GGA AC-3' R 5'-ACC ACC TCT CTG TGC AAT CC-3'
Bcl2	NM_016993	F 5'-AAG CTG TCA CAG AGG GGC TA-3' R 5'-CAG ATG CCG GTT CAG GTA CT-3'
Bid	NM_022684	F 5'-ACC GTG ATT TCC ACC AAG AG-3' R 5'-TGG CAA TGT TGT GGA TGA CT-3'
MyoD1	NM_017115	F 5'-CGA CTG CCT GTC CAG CAT AG-3' R 5'-GGA CAC TGA GGG GTG GAG TC-3'
MyoG	NM_176079	F 5'-CAG CAT CAC GGT GGA GGA TA -3' R 5'-CAC TTA AAA GGC CCC TGC TG -3'
Vegfa	NM_001110333	F 5'-TCA TGC GGA TCA AAC CTC AC-3' R 5'-TCT TGC GCT TTC GTT TTT GA -3'
BMP7	NM_001191856.2	F 5'-ATT TGA CAT CAC AGC CAC CA -3' R 5'-GGG AGC TCC ACT TGG ACA AC -3'
IL6	NM_012589.2	F 5'-GAG GCT GGC ATC CAT GTC TT -3' R 5'-CCT CCA GCT CCT CAC ATT CC -3'
IL15	NM_013129.2	F 5'-ACA GCG ATG ATG CAC TGT CA -3' R 5'-GCA TTG GAA GTT GGG GTA GG -3'
Apln	NM_031612	F 5'-CTC TCC TTG ACT GCC GTG TG -3' R 5'-AAA GGC ATG GGT CCC TTA TG-3'
Sesn1	NM_001106396	F 5'-TCC TTC ACA TTT GGC TGT GG -3' R 5'-TTC AAA CCT CGA GGC CAT CT -3'
SDF1	NM_022177	F 5'-CCT GCC GAT TCT TTG AGA GC -3' R 5'-TTT CGG GTC AAT GCA CAC TT -3'
SPARC	NM_012656.1	F 5'-GAG GGC AAC AAC CTC CTC AC -3' R 5'-CGA GTG GTG CAA TGT TCC AT -3'
Visfatin	NM_177928.3	F 5'-TGT TCC TGA GGG CTC TGT CA -3' R 5'-TCT GCC GCT GGA ACA GAA TA -3'
Musclin	NM_207612.2	F 5'-CTT GCA GCT AAG CTC CTG CT -3' R 5'-TGG AAC TGG AGA GAC GGT TT -3'
Dcn	NM_024129	F 5'-GGG AGC TCC ACT TGG ACA AC-3' R 5'-ATT TGC CAA TAC CGG ACA GG-3'
PPIA	NM_017101.1	F 5'-GGT GAC TTC ACA CGC CAT AA-3' R 5'-CTT CCC AAA GAC CAC ATG CT-3'