Growth Hormone & IGF Research 82 (2025) 101663

Contents lists available at ScienceDirect

Growth Hormone & IGF Research

journal homepage: www.elsevier.com/locate/ghir

Injection of lentiviral vectors expressing GH and IGF1 increases body and muscle mass in male rats

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ARTICLE INFO

Keywords:
Growth hormone
Insulin-like growth factor-1
Lentiviral vectors
Muscle

ABSTRACT

The aim of this study was to increase growth hormone (GH1) and insulin-like growth factor-1 (IGF1) levels in rat muscle indirectly through the transduction of C2C12 cells via lentivectors and to compare their effects on muscle mass. The coding sequences of GH1, IGF1, and GH1-IGF1, linked by the 2 A self-cleaving peptide to enable polycistronic expression, were synthesized, cloned and inserted into the pCDH vector. Recombinant pseudo-lentiviruses containing our target genes were produced in HEK293T cells. C2C12 cells were transduced with pseudo lentiviruses and selected through resistance to puromycin antibiotics. The expression of the GH1 and IGF1 genes at the mRNA and protein levels was verified by RT–PCR and Western blotting, respectively. The recombinant pseudo lentiviruses and transduced C2C12 cells were injected into the tibialis anterior (TA), gastrocnemius and quadriceps muscle groups of eight-week-old rats. The body weights of the rats were measured weekly for eight weeks after the injection. The leg weight and histology of the muscle after eight weeks were also measured. The results revealed the expression of the GH1 and IGF1 genes at the mRNA and protein levels in C2C12 cells. An increase in both hormones, either directly or indirectly through C2C12 cells, increased the animal body weight, leg weight, and muscle fibre size after eight weeks. The direct and indirect transfer of IGF1 increased body weight, leg weight and muscle fibre size more than did the direct or indirect transfer of GH1. In the direct transfer groups, the body weight was greater than that in the indirect transfer groups after eight weeks. We demonstrated that nonpituitary secretion of GH1 mimicked some physiological effects of pituitary GH1 in a rat model. Further investigations are needed to study other possible effects of extra copy of GH1 and IGF1 genes over a longer period on other tissues.

1. Introduction

The complex somatotropin system controls animal growth, in which growth hormone (GH1) and insulin-like growth factor-1 (IGF1) are important. They are responsible for some physiological processes and growth after birth. GH1 acts both directly through its receptors and indirectly through the induced production of IGF1 [1]. The GH1 and IGF1 genes are important key growth drivers and play essential roles in the growth process in animals [2,3]. Therefore, these genes are suitable candidates for improving the economic traits of growth and increasing muscle mass in animals. The most important biological role of GH is postnatal growth control, and the effects of this hormone on the development of some tissues, such as bone, muscle, and adipose tissues, have been identified. Both IGF1-dependent and independent effects have been described by scientists [4,5]. The secretion of GH from the pituitary gland stimulates the secretion of IGF1 from the liver and other tissues in the body. The presence of topical IGF1 in tissues can accelerate the effects of GH on growth and metabolism [6]. The most compelling evidence to prove the vital role of IGF I in the embryonic and postnatal developmental stages of animals was obtained from animals with IGF1 deficiency. These animals not only have low weight at birth (~ 60 % of normal newborn weight) but also have severe muscle atrophy; most of

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https://doi.org/10.1016/j.ghir.2025.101663
Received 24 October 2024; Received in revised form 20 August 2025; Accepted 21 August 2025
Available online 28 August 2025

them (90 %) are underweight and infertile and die after birth [7]. The transfer of external genes into cells can be performed via nonviral and viral methods. Nonviral systems include all physical and chemical systems but not all viral systems. The proficiency of nonviral methods is lower than that of viral systems in terms of gene transfer [8]. Lentiviral vectors, which have been considered by genetic engineering researchers in recent years, have the advantages of high-efficiency infection of dividing and nondividing cells, long-term stable expression of a transgene, low immunogenicity and the ability to accommodate larger transgenes [8]. They are used as powerful tools for the transfer of genes into host cells [9]. The aim of this study was to investigate the effects of injecting lentiviral vectors expressing GH1 and IGF1 into the muscle of male rats. Specifically, we sought to determine whether such injections could lead to measurable changes in body and muscle mass. While previous studies have demonstrated the potential of GH1 and IGF1 to promote muscle growth, the direct effects of lentiviral-mediated gene delivery in vivo remain poorly understood. Here, we used lentiviral vectors to deliver GH and IGF1 transgenes and assessed their impact on muscle and body mass in a rat model.

2. Methods and materials

2.1. Design of the study

In this study, three pseudolentiviruses carrying GH1, IGF1 and GH1-IGF1 were produced. These viruses, both directly and indirectly after transduction into C2C12 cells, were injected into the tibialis anterior (TA), gastrocnemius and quadriceps muscle groups of 8-week-old rats.

2.2. Design of the construct

The sequences of the *Homo sapiens* insulin-like growth factor 1 IGF1 and *Homo sapiens* growth hormone (GH1) genes were retrieved from NCBI, including the signal peptide (accession numbers NM_001111283.1 and NM_000515.3, respectively). The viral self-cleaving 2 A peptide (p2A) sequence was added between two genes for simultaneous overexpression of both IGF1 and GH1 genes. This sequence was synthesized and inserted into the pUC57 plasmid by GenScript Company (Piscataway, USA). Afterward, competent cells (*E. coli* DH5α) were transformed with the recombinant pUC57 plasmid. The transformed bacteria were cultured on LB agar supplemented with ampicillin at 37 °C for 24 h. Plasmids were extracted from the colonies that appeared on LB plates via the Plasmid Extraction Miniprep Kit (Qiagen, Germany), digested with *Hin*dIII and *Eco*RI restriction enzymes, and then electrophoresed on a 1 % agarose gel. Finally, the inserts were sequenced.

2.3. Lentiviral vector construction

The lentivector pCDH was purchased (SystemBio: CD513B-1) as a backbone vector. pCDH contains two promoters, CMV and EF1. The EF1 promoter mediates the co-expression of GFP and puromycin resistance, which are mammalian selection markers that are released as monoproteins via the T2A self-cleavage peptide. The GH1-P2A-IGF1 segment, which was previously cloned and inserted into pUC57, was excised and subcloned and inserted into the pCDH vector. GH1 and IGF-1 were expressed in the recombinant lentiviral vectors (pCDH-GH1 and pCDH-IGF1) as previously described [10,11]. In addition, a third construct named pCDH-GH1-IGF1 was generated, which mediated the co-expression of GH1 and IGF1. To confirm the cloning procedure, recombinant pCDH-GHI-IGF1 was digested and then sequenced via *Xba*I and *Bam*HI.

2.4. Cell cultures

HEK293T cells and mouse myoblast C2C12 cells (ATCC CRL-1772) were obtained from the Pasteur Institute of Iran (Tehran, Iran) and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, USA) at 37 °C in a humidified 5 % CO_2 atmosphere.

2.5. Production of pseudo lentiviruses

The lentiviral vectors (pCDH-GH1, pCDH-IGF1 and pCDH-GH1-IGF1) were transfected with two plasmids (psPAX2 and pMD2. G) in HEK-293 T cells via the standard calcium phosphate precipitation method according to the Pro. Trono laboratory protocol with some modifications [12]. On day one, 5 × 106 HEK-293 T cells were seeded in a 10 cm plate in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10 % fetal bovine serum (FBS) (Gibco, USA). On the second day, 21 μg of transfer vector, 21 μg of psPAX2 vector and 15 μg of pMD2. The G vector was mixed with the transfection buffer and added dropwise to the cells. The transfection medium was replaced with fresh medium within 14 h post-transfection. GFP expression was observed by fluorescence microscopy after 24 h. The packaged recombinant lentiviruses were harvested from the supernatants of the cell cultures at 48 h post-transfection and were subsequently centrifuged at 2000 rpm at 4 °C for 5 min, after which the supernatants were filtered through a 0.22 μm filter. The recombinant virus was stored at −70 °C for subsequent experiments.

2.6. Transduction of C2C12 cells

Myoblast C2C12 cells were cultured at a low confluency of 30–40 % in a 6-well plate. On the following day, the cells were transduced with recombinant lentiviral and control vectors at an MOI of 30. After 24 h, the transduction media was replaced with fresh DMEM supplemented with 10 % FBS. The transduced cells were passaged every three days. After 72 h, cell transduction was assessed with a fluorescence microscope. The fluorescence properties of GFP were examined under a LABOMED® Model T121100 fluorescence microscope (Labo, USA). The obtained images were assessed by ImageJ software [13].

2.7. Selection of transduced C2C12 cells with puromycin

With the aim of generating stable transgenic cells, in which the GH1 and IGF1 genes were introduced into the genome, as well as the removal of cells that were not transfected after transmission, antibiotic treatment was performed by placing infected cells in the selected medium supplemented with puromycin 96 h after infection. The cells were selected 72 h after transduction with 1.5 μg/ml media containing puromycin for five days.

2.8. Validation of GH1 and IGF1 expression in C2C12 cells via RT–PCR

Total RNA was extracted from transduced and non-transduced C2C12 cells using the RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's protocol. To eliminate any contaminating vector DNA, RNA samples were treated with DNAse I prior to reverse transcription. Complementary DNA (cDNA) was synthesized using the QuantiNova Reverse Transcription Kit (Qiagen, Germany) according to the manufacturer's instructions. RT-PCR was performed using gene-specific primers for GH1, IGF1, and β-actin (see Table 1 for primer details). The RT-PCR conditions were as follows: 95 °C for 5 min; 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. The PCR products were separated on a 1.5 % agarose gel and stained with DNA Safe Stain (SinaClon, Iran). Gel images were visualized using a UV transilluminator (SABZ Biomedical, Iran), and the integrated optical density (IOD) of each band was measured using ImageJ software [14].

Table 1
Characteristics of Primers Used for RT-PCR Analysis.

Name of Gene	Primer Sequence (5′ → 3′)	Fragment Size (bp)	Ta (°C)
IGF1	F: AGGAGGCTGGAGATGTATTG R: TCGTGTTCTTGTTGGTAGATG	127	58
β-actin	F: AGCCTCGCCTTTGCCGA R: CTGGTGCCTGGGGCG	172	58
GH1	F: TAGAATGGCTACAGGCTCC R: GCTTCTTCAAACTCCT	183	58
GH1-IGF1	F: TCTAGAATGGCTACAGGCTCCCGG R: GCGGCCGCTCTTTTTGCC	1114	56

2.9. Western blot analysis

The recombinant C2C12 cells were collected 72 h after transduction. The cells were lysed in 200 µl of RIPA buffer (Thermo Fisher Scientific, USA) supplemented with protease inhibitor. The cell lysates were centrifuged at 11000 rpm for 12 min at 4 °C, and the supernatants were collected [11]. The protein concentrations of the supernatants were determined with a BCA protein assay kit (Thermo Fisher Scientific, USA). Protein lysates (30 µg/lane) were loaded onto 12 % SDS–PAGE gels with protein markers and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, USA) [11]. The membranes were blocked with 5 % nonfat dried milk, and immunoblotting was performed with primary antibodies against IGF1, GH1 (Santa Cruz, USA) and β-actin (Santa Cruz, USA). Proteins of interest were detected with HRP-conjugated rabbit polyclonal IgG (Santa Cruz, USA) as the secondary antibody. Finally, the protein bands were visualized with a chemiluminescence (ECL) reagent, and the integrated optical density (IOD) of each protein band was measured [15].

2.10. Animals and injection procedure

In this study, 60 adult male Wistar rats weighing 150–180 g were purchased from the pasture institute. The animals were given adequate access to food and water at a temperature of 21 ± 3 °C and a 12 h dark/light cycle before and during the study.

The animals were randomly divided into ten groups ($n = 6$): the C2C12 group (injection of PBS-C2C12), 2 groups (injection of the native pCDH vector or C2C12 cells transduced with pCDH), 2 GH1 groups (injection of pseudolentiviruses carrying the GH1 gene and injection of C2C12 cells expressing/overexpressing the GH1 gene), 2 IGF1 groups (injection of Pesodolentiviruses carrying the IGF1 gene and injection of C2C12 cells expressing/overexpressing the IGF1 gene), two GH1-IGF1 groups (injection of pseudoliviruses carrying the GH1 and IGF1 genes and injection of C2C12 cells expressing/overexpressing the GH1 and IGF1 genes) and the untreated group (without injection). All methods were carried out in accordance with relevant guidelines and regulations. All animal procedures were complied with ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) and carried out in accordance with Guidance on the operation of the Animals (Scientific Procedures) Act 1986 and associated guidelines. These procedures approved by Ferdowsi University of Mashhad's ethical committee, permit number: 3/29769.

The tibialis anterior (TA), gastrocnemius and quadriceps muscle groups were injected with 1×10^7 lentivirus particles diluted in 75–100 µl of phosphate-buffered saline (PBS) or transduced C2C12 cells (3×10^6). The animals were weighed weekly for eight weeks after the injection.

2.11. Histological analysis

The animals were sacrificed eight weeks after the injection via an overdose of ketamine and xylazine. The muscle tissues were dissected, washed in PBS, and frozen rapidly in liquid nitrogen. A block of the muscles, 1.5 cm in length, was embedded in PBS, and then 10 µm cross-sections were prepared using a cryostat (Leica, Germany) and stained with hematoxylin and eosin. The stained sections were assayed and photographed using an optical microscope. Photographs were analyzed by AxioVision software (Version 4.8.2.0), and the fibre cross-sectional areas (CSAs) were measured.

2.12. Statistical analysis

All the experiments were carried out in triplicate, and the results are expressed as means ± standard deviation (SD). Statistical differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for post-hoc comparisons, using SPSS software (version 25). Differences were considered statistically significant at $p < 0.05$. In the figures, different letters (e.g., "a," "b," "c") are used to indicate statistically significant differences between groups. Groups labeled with the same letter are not significantly different, while groups labeled with different letters are significantly different at $p < 0.05$. This method was chosen to provide a clear and concise representation of group comparisons, particularly when analyzing multiple groups simultaneously.

3. Results

3.1. Confirmation of the IGF1-P2A-GH1 construct

The transfer vector, which can express four proteins from two mRNAs, is shown in Fig. 1. The results of the digestion of the construct (IGF1 - P2A- GH1) are shown in Fig. 2. A 1200 bp segment was the expected size related to IGF1-P2A-GH1.

3.2. Confirmation of pCDH-IGF1-P2A-GH1 cloning

The successful cloning of the pCDH-IGF1-P2A-GH1 construct was confirmed by restriction digestion and gel electrophoresis, as shown in Fig. 3. The undigested plasmid (Lane 1) appeared as a single band at approximately 10,000 bp, corresponding to the full size of the pCDH-IGF1-P2A-GH1 plasmid. Following restriction enzyme digestion, the plasmid yielded two distinct bands in Lane 2: a 1200 bp fragment, corresponding to the IGF1-P2A-GH1 insert, and a larger fragment of approximately 8800 bp, representing the plasmid backbone. The presence of the 1200 bp band confirmed the successful insertion of the IGF1-P2A-GH1 cassette into the pCDH vector. The transfection of HEK293T cells was assayed via the expression of GFP with a fluorescence microscope 24, 48 and 72 h after transfection, the results of which are shown in Fig. 4. Approximately, more than 80 % of the HEK293T cells were transfected with the lentivirus pCDH-IGF1-P2A-GH1.

3.3. Transduced C2C12 cells

After 72 h, the transduction efficiency of C2C12 cells with pseudolentiviruses carrying GH1-IGF1, GH1, and IGF1 was assessed. GFP expression, indicative of successful transduction, was observed under a fluorescence microscope (Fig. 5). At a multiplicity of infection (MOI) of 20, more than 90 % of the transduced cells were found to be GFP-positive, as confirmed by fluorescence-activated cell sorting (FACS) analysis. These results demonstrate the high efficiency of the pseudolentiviral system in delivering the target genes (GH1 and IGF1) into C2C12 cells.

3.4. Selection of transduced C2C12 cells with puromycin

To increase the purity of transduced cells, C2C12 cells were subjected to puromycin selection after transduction with pseudolentiviruses carrying GH1-IGF1, GH1, or IGF1. As shown in Fig. 6,

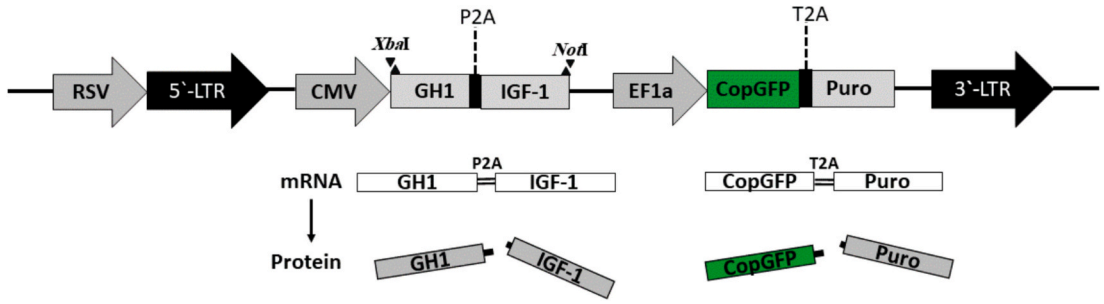

Fig. 1. Polycistronic third-generation lentiviral vectors. The RSV promoter before the 5-LTR is cloned is a Tat-independent and third-generation lentiviral vector. RSV results in complete viral genome transcription during the packaging process. Two transcripts derived from the CMV and EF1 promoters. The introduction of P2 A and T2 A self-cleavage peptides ensures the release of each protein. This polycistronic lentivirus can overexpress four proteins simultaneously in transduced cells. RSV; Rous sarcoma virus, LTR; Long Terminal Repeat, CMV; Cytomegalovirus, EF1a; Elongation Factor-1a, P2 A; Porcine Tescho virus-1 2 A, and T2 A; Thosea asigna virus 2 A.

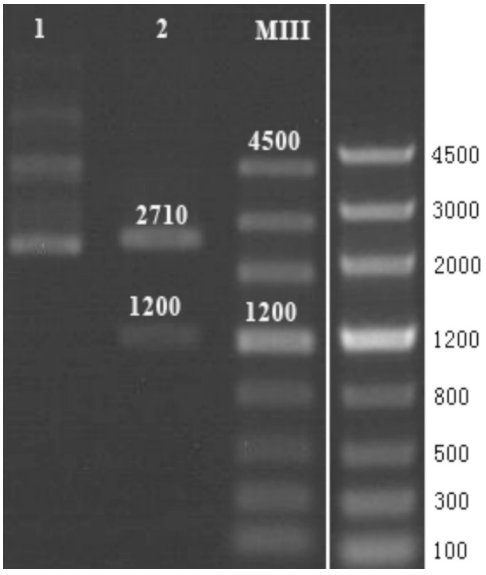

Fig. 2. The pUC57 plasmid was digested with *Eco*RI and *Hind*III restriction enzymes. The 1200 bp segment is related to the IGF1-P2A-GH1 sequence. Lane 1: Undigested plasmid containing the IGF-1-P2A-GH1 construct. Lane 2: Digested plasmid showing the expected 1200 bp fragment (IGF-1-P2A-GH1 insert) and additional fragments corresponding to the plasmid backbone. Lane M: DNA ladder (marker) with bands corresponding to the indicated sizes (in base pairs).

puromycin treatment effectively eliminated non-transduced cells, resulting in a highly enriched population of GFP-positive cells. This step was critical for ensuring the homogeneity of the transduced cell population in subsequent experiments.

3.5. Analysis of GH1 and IGF1 expression in C2C12 cells via RT–PCR

The RT-PCR results confirmed the expression of GH1 and IGF1 in C2C12 cells transduced with the pseudo-lentiviral constructs. Distinct bands corresponding to GH1 (183 bp) and IGF1 (127 bp) were observed in the transduced cells, while no amplification was detected in the non-transduced control cells (Fig. 7). The housekeeping gene β-actin (172 bp) was used as an internal control to normalize the results. To ensure the rigor of the RT-PCR analysis, the following controls were included: The Non-Reverse Transcriptase Control (−RT), which showed no amplification, confirming the absence of DNA contamination; the No Template Control (NTC), which also showed no amplification, ruling out reagent or primer contamination; and the Empty Vector Control, which showed no amplification of GH1 or IGF1, confirming the specificity of the primers. These results demonstrate that the pseudo-lentiviral system effectively delivered and expressed GH1 and IGF1 in C2C12 cells, confirming the functional integration of the recombinant lentiviral vector into the host genome.

3.6. Analysis of GH1 and IGF1 expression in C2C12 cells via Western blot analysis

To confirm the expression of GH1 and IGF1 proteins in transduced C2C12 cells, Western blot analysis was performed. The immunoblotting results revealed that GH1 and IGF1 proteins were specifically produced in C2C12 cells transduced with the respective constructs (Fig. 8). Using densitometry analysis of band intensities from SDS-PAGE, the concentrations of recombinant GH1 and IGF1 proteins were estimated to be in the range of 0.23–0.30 mg/ml. ImageJ software was utilized for this quantification. β-actin was included as a loading control, and its corresponding bands are now presented in Fig. 8.

4. Animal study

4.1. Body weights of the animals in the groups that received transduced cells

In the transgenic cell recipient groups, the body weights of all the groups increased over time. From week five onwards, this weight gain was significantly ($p < 0.009$) faster than before. The greatest weight gain occurred in the transgenic cell recipient group with two hormones simultaneously, followed by the transgenic cell with IGF1 (Fig. 9).

4.2. Body weights of the animals in the groups receiving pseudo-viral vectors

In the animal groups that received pseudolentiviruses, the body weights increased over time. Weight gain from week four onwards was significantly ($p < 0.031$) greater in the groups than before. The greatest weight gain occurred in the GH1-IGF1 group, followed by the IGF1 group (Fig. 10).

4.3. Comparison of the body weights of the animals that received transgenic C2C12 cells with those of the recipients of pseudovirus

A comparison of the body weights of the animals in the groups receiving transgenic C2C12 cells with the body weights of the animals in the groups receiving pseudovirus revealed that the highest weight gains occurred in the animals that received pseudovirus carrying GH1-IGF1 at week eight, followed by those receiving IGF1. Direct injection of the virus caused more weight gain than did injections of transgenic cells (Fig. 11).

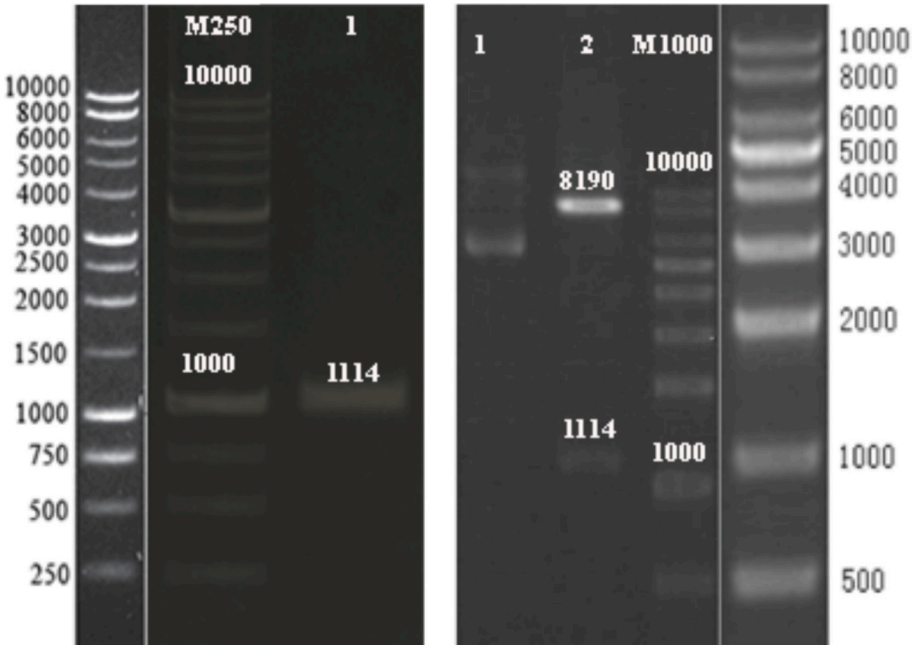

Fig. 3. Construction of the IGF-1-P2A-GH1 recombinant lentivirus vector. Left) Cloning PCR for pCDH-IGF-1-P2A-GH1. Lane 1: The PCR product was amplified using specific primers to confirm the presence of the IGF-1-P2A-GH1 insert. Single band of approximately 1200 bp was observed, corresponding to the expected size of the IGF-1-P2A-GH1 cassette. Lane M: DNA ladder (marker) with bands corresponding to the indicated sizes (in base pairs). Right) Restriction digestion of the pCDH-IGF-1-P2A-GH1 plasmid with *XbaI* and *NotI* enzymes. The digestion yielded two fragments: a 1200 bp band, corresponding to the IGF-1-P2A-GH1 insert, and a larger band of approximately 8800 bp, representing the linearized plasmid backbone. The results confirm the successful cloning of the IGF-1-P2A-GH1 cassette into the pCDH vector. Lane 1: Undigested pCDH-IGF-1-P2A-GH1 plasmid. Expected size: ~10,000 bp (full plasmid). Lane 2: Digested pCDH-IGF-1-P2A-GH1 plasmid with XbaI and NotI. Expected sizes: 1200 bp (IGF-1-P2A-GH1 insert) and ~ 8800 bp (plasmid backbone). Lane M: DNA ladder (marker) with bands corresponding to the indicated sizes (in base pairs).

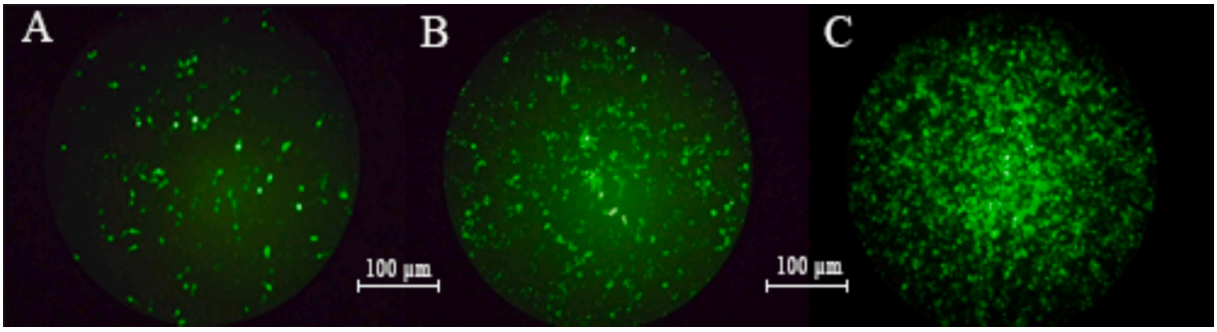

Fig. 4. HEK-293 T cells were transfected with pCDH-IGF1-2A GH1 to generate viral particles. A) HEK-293 T cells at 24 h after transfection, B) HEK-293 T cells at 48 h after transfection and C) HEK-293 T cells at 72 h after transfection.

4.4. Comparison of leg weight in transgenic cell recipients and virus recipients

After eight weeks, leg weight significantly increased in the GH1-IGF1 and IGF1 groups. There was no significant difference in leg weight between the transgenic cell and virus-receiving groups (Fig. 12).

4.5. Histological analysis of muscle tissue

Histological analysis of the muscle tissue was performed eight weeks after the injection of lentivirus and transduction of C2C12 cells. Fluorescence microscopy revealed the presence of GFP-positive cells in the muscle sections, indicating that the transduced cells and lentivirus remained localized in the injected area (Fig. 13). While the GFP signal was observed, some background autofluorescence from the extracellular matrix was also noted. This suggests that the transduced cells were present in the muscle tissue, but further controls and molecular analyses (e.g., RT-PCR) would be beneficial in future studies to confirm the expression levels of GH1 and IGF1 in the treated muscles.

4.6. Muscle fibre size and growth

Hematoxylin and eosin (H&E) staining of the muscle fibres showed a noticeable increase in fibre cross-sectional area (CSA) in all experimental groups compared to the untreated control group (Figs. 14 and 15). The most significant increase in muscle fibre size was observed in the groups receiving combined GH1 and IGF1 treatment, followed by the groups receiving IGF1 alone. These results suggest that the overexpression of GH1 and IGF1, either through lentiviral injection or transduced C2C12 cells, promoted muscle growth in the treated animals. There were no significant differences between the groups receiving the cells or the pseudo viruses (Fig. 16).

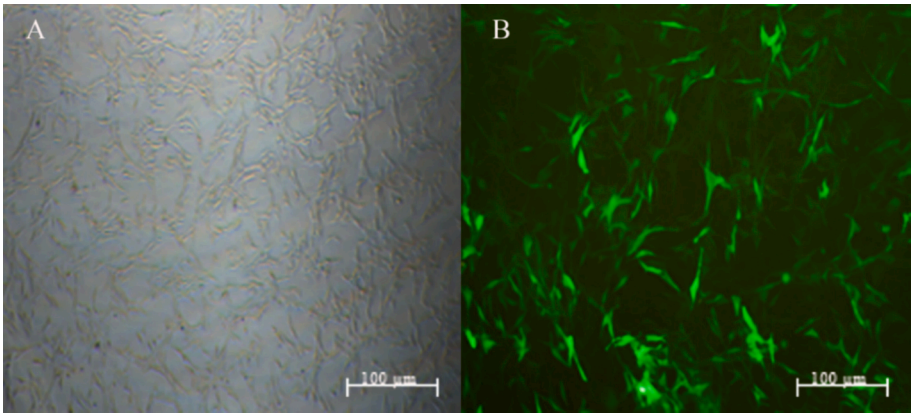

Fig. 5. C2C12 cells were transduced with pseudo lentiviruses carrying GH1 and IGF-1. A) pre-transduction: C2C12 cells prior to transduction, showing the normal morphology and growth of the cells. and B) post-transduction: transduced C2C12 cells with pseudo lentiviruses. as evidenced by (state the evidence, e.g., changes in cell morphology, expression of a reporter gene, or other observable effects). Scale bars: 100 µm (Scale bar: 100 µm (10× magnification)).

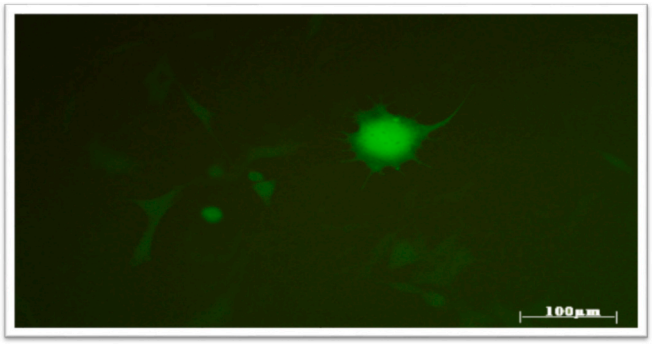

Fig. 6. The selection of transduced cells was performed with puromycin. The image shows C2C12 cells after puromycin selection. Scale bar: 100 µm (40× magnification).

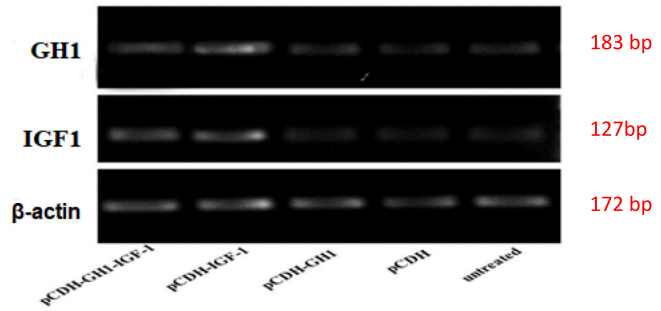

Fig. 7. RT–PCR analysis of GH1 and IGF1 in C2C12 cells.

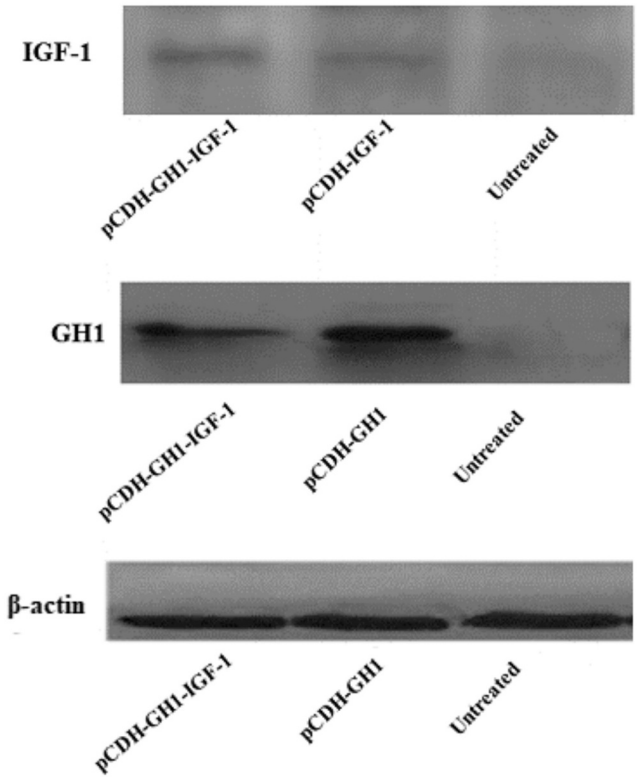

Fig. 8. Western blot images of GH1 and IGF1 protein expression in C2C12 cells.

5. Discussion

Since many animal genetic engineering studies over the past decades have focused on the use of the GH1 and IGF1 genes to improve and accelerate the growth and genetic modification of animals, this study was performed to increase GH1 and IGF1 hormone levels in rat muscle, directly through the injection of pseudolentiviruses and indirectly through the injection of transduced C2C12 cells, to compare their effects on muscle mass. The in vitro and in vivo results of this research revealed that GH1 and IGF1 gene transfer through pseudo lentiviruses and the transduction of C2C12 cells into the muscle of the hind limb of rats were possible and could increase the size and proliferation of muscle cells.

Compared with control mice, transgenic mice with human IGF1 and high expression of the gene were almost 30 % heavier [16]. Additionally, IGF1, which is independent of growth hormone, stimulates the hypertrophy of skeletal muscle [17,18].

It has been reported that GH1 causes hypertrophy of the leg muscle, which also affects protein synthesis. It is believed that GH1 does not affect the proliferation of cells but that IGF1 increases both cell proliferation and protein synthesis. Therefore, GH1 and IGF1 act directly in muscle hypertrophy [5,19]. Many cells are stimulated by IGF1 and exhibit myogenic responses. In addition to fibroblasts, IGF1 stimulates myogenic responses in chondrocytes, osteoblasts, keratinocytes, follicular thyroid cells, smooth muscle cells, skeletal muscle cells, nerve cells, mammalian epithelial cells, erythroid cells, oocytes, granulose cells and several cancer cell lines [20,21].

In addition to its functional role as a stimulant of cell proliferation,

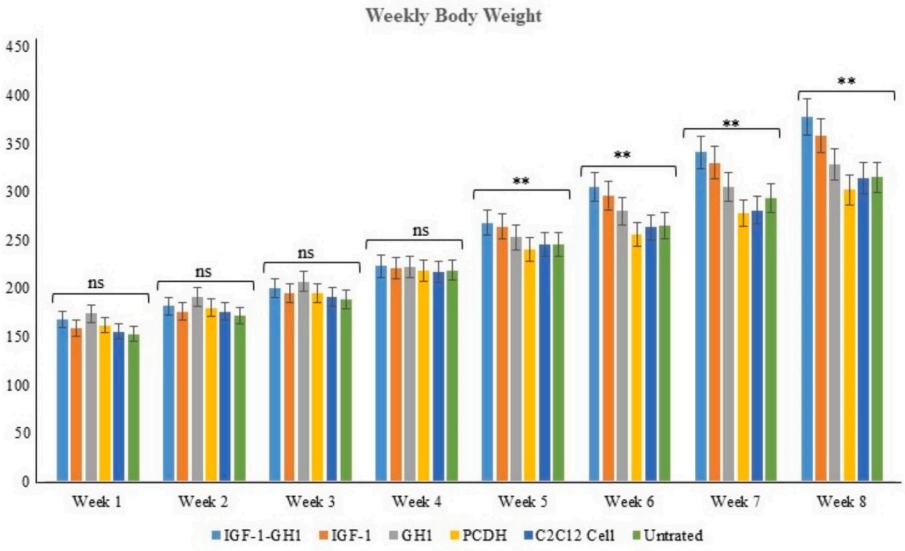

Fig. 9. The body weights of the animals injected with transduced cells. (Significance levels are indicated as follows: ** indicates significance at the 1 % level, and ns indicates not significant.)

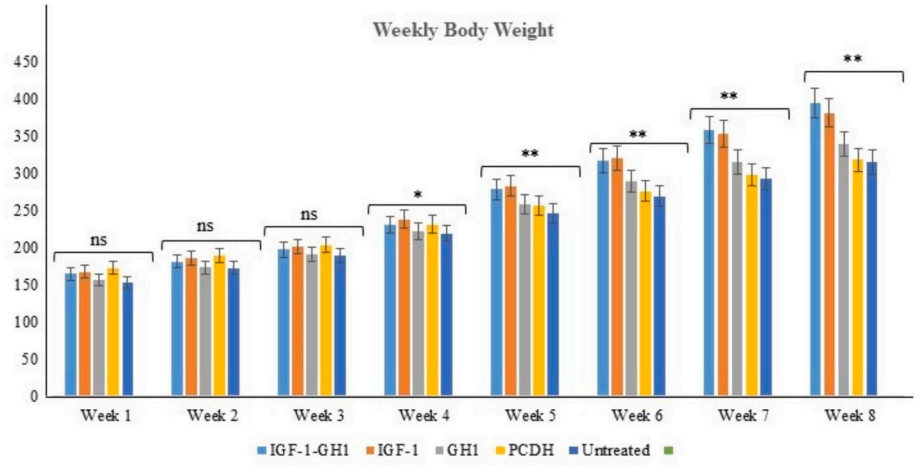

Fig. 10. Body weights of animals injected with viruses. (Significance levels are indicated as follows: ** indicates significance at the 1 % level, * indicates significance at the 5 % level, and ns indicates not significant.)

IGF1 can act as an inhibitory factor in apoptosis [20]. Thus, understanding the molecular mechanism of these genes in specific cells and tissues can be useful in livestock breeding.

A strategy that has been of interest to researchers over the last few years is the use of two distinct promoters within the structure of a vector [22]. Since the use of two vectors, especially when transmission efficiency is low, can lead to a mismatch between the amount of transient genes received by the cells and make it difficult to evaluate the results, Yu et al., 2003, introduced a group of carriers called polycistronic, which has several advantages, such as the application of all molecular components within a conventional system and the possibility of simultaneous expression of two genes in a carrier [23,24].

Numerous studies have concentrated on the simultaneous use of two promoters in the same or different directions in polycistronic vectors in which the expression of the two genes is contemporaneous. Accordingly, in this study, two-cystronic vectors expressing the GH1 and IGF1 genes were constructed based on a lentiviral vector.

Microscopic observations after the transfection of HEK293T cells with lentivectors carrying GH1 and IGF1 via the GFP reporter gene revealed more than 80 % expression of the GFP reporter gene and successful production of the recombinant virus. The virus production in the present study was based on the methods used in the research of Dastpak et al. (2014) and Alizadeh et al. (2015). After the transfection of HEK293T cells, more than 70–80 % of the cells produced a virus similar to that in the present study [25,26]. Previous studies have shown that the transfection efficiency is greater in the HEK293T cell line than in other lines [27]. HEK293T cells have an epithelial morphology and are among the best cell lines for the expression of recombinant genes and the production of viruses. Almost all viral vectors are capable of binding, transducing, and integrating into their genome. After being integrated into the genome, they become part of its genome and are expressed, similar to other cell genes, and produce proteins [28].

The results of the transfection of HEK293T cells showed that the transfection capability of HEK293T cells was high, and viruses carrying the GH1 and IGF1 genes were successfully produced. The results obtained from fluorescence microscopy, as shown in Fig. 5, revealed that 70 % of the C2C12 cells were transduced with viruses. C2C12 cells are a good model for muscle cells.

In 2011, Varma and colleagues investigated the effects of four types of promoters on the transduction efficiency of hematopoietic stem cells.

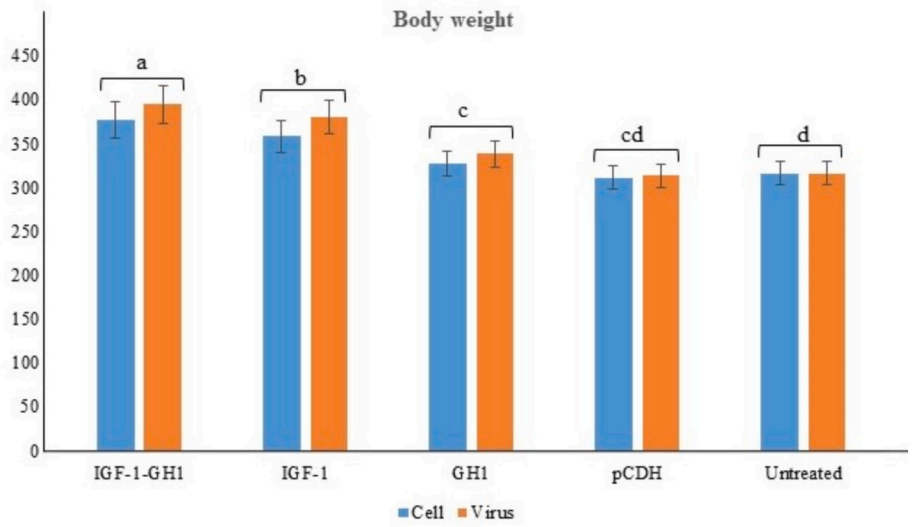

Fig. 11. Comparison of animal body weights 8 weeks after the injection of viruses and transduced cells (for all groups with the same letter, the difference between the means is not statistically significant). If two groups have different letters, they are significantly different (p-values<0.05).

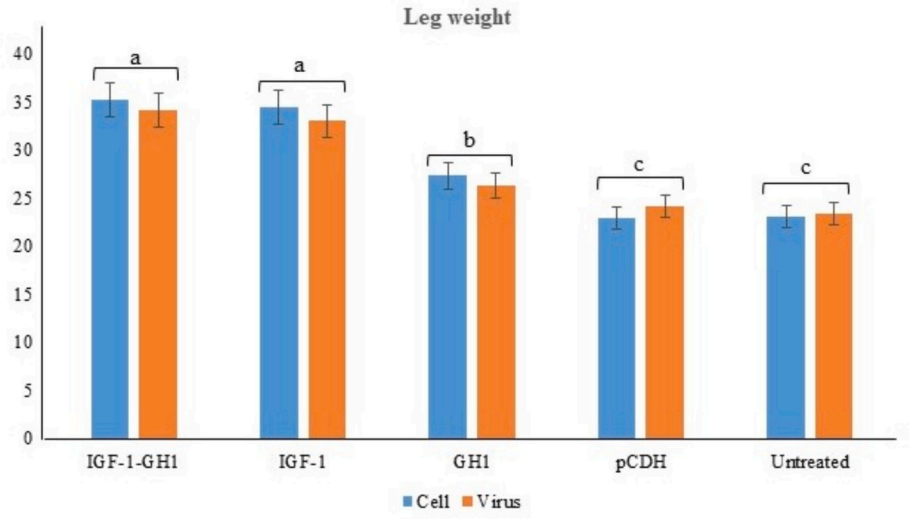

Fig. 12. Comparison of the leg weights of the animals 8 weeks after virus injection and cell transduction. (For all groups with the same letter, the difference between the means is not statistically significant. If two groups have different letters, they are significantly different (p-values<0.05).

These studies revealed that the CMV and EF-1 promoters presented the highest GFP expression level and, consequently, the highest transduction rate, whereas the SV40 and UBC promoters presented the lowest GFP expression level and thus the lowest transduction rate in hematopoietic stem cells [29].

Fitzmans et al. (2002) also used the CMV promoter as an appropriate promoter in studies of gene therapy and various eukaryotic cells [30]. In the present study, consistent with the results of the above investigations, the reporter gene under the EF-1 promoter was expressed during the transfection process in the HEK293T cell line, and transduction in the C2C12 cell line resulted in a high expression level of GFP, resulting in a relatively high rate of gene expression.

The observation of GFP in C2C12 cells indicated the proper expression of the construct in C2C12 cells. Additionally, RNA and protein production was confirmed by subsequent RT–PCR and Western blotting, which revealed an increase in the expression of GH1 and IGF1 in C2C12 cells. The presence of the poromycin resistance gene was another characteristic of this expression vector, which provided the opportunity for the selection and purification of transduced cells. Transduction was confirmed by fluorescence microscopy, as shown in Fig. 6. The transduced cells were selected after being treated with this antibiotic. The confirmed effect of IGF1 on the stimulation of DNA synthesis and cell division has been shown extensively in 3 T3 fibroblasts (a cell type that does not produce IGF1) [31]. IGF1, a muscle growth factor, is closely related to GH1 and plays a mediating role in the effects of growth hormones. The effects of the coadministration of recombinant GH1 and IGF1 on the growth and proliferation of breast epithelial cells were investigated, and the results indicated that IGF1 promoted cell growth and proliferation. In contrast, in cells that received recombinant GH1 because the GH1 receptor was not present before, the cells were treated with GHR and STAT5 and then treated with GH1, and the authors reported that cell growth and proliferation did not change and concluded that cell proliferation was dependent on IGF1, not the direct effect of GH1 [32].

Zarratigui et al. (2002) used an adenoviral vector to transmit the IGF1 gene to study the effect of IGF1 on hepatic activity in a rat model of liver cirrhosis. They reported that, in rats receiving intramuscular injection of viruses, the body weight and leg muscle increased compared

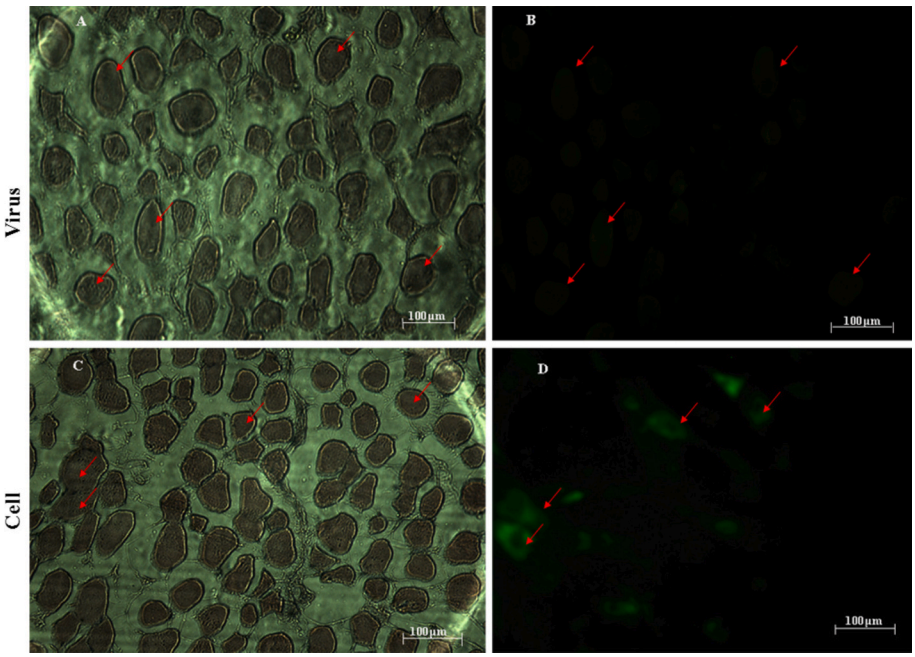

Fig. 13. Fluorescence microscopy of muscle sections 8 weeks after injection of lentivirus and transduction of C2C12 cells. GFP signal indicates the presence of transduced cells in the muscle tissue.

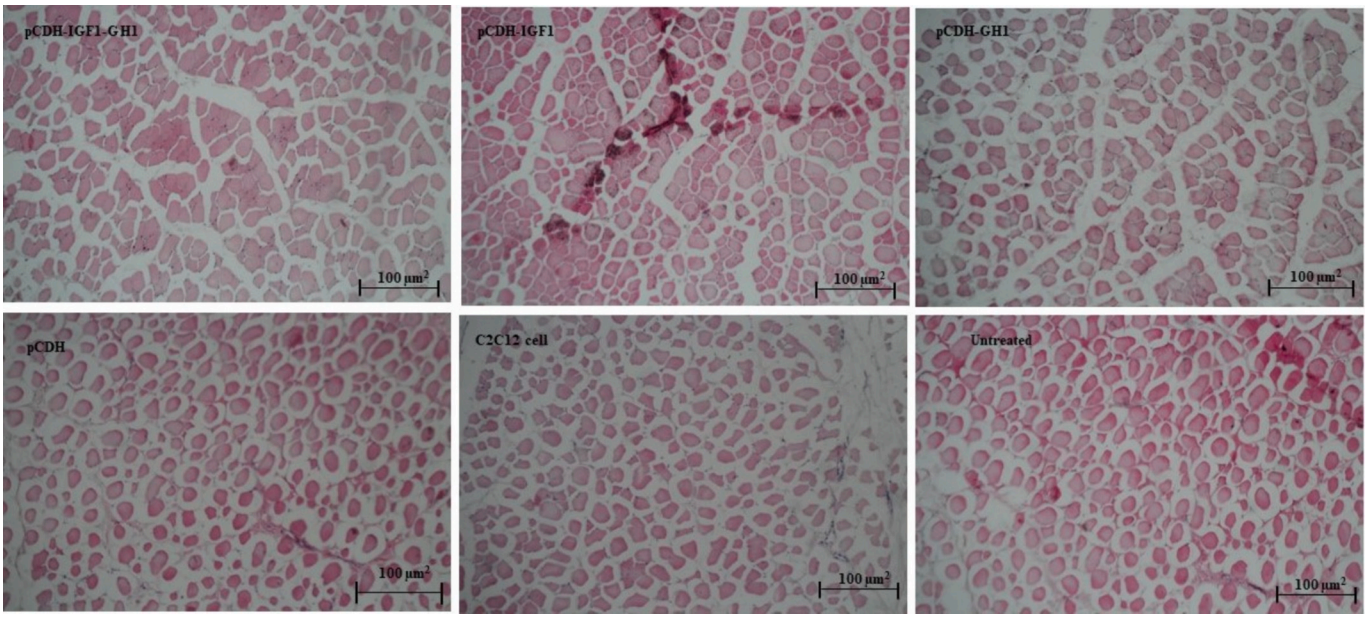

Fig. 14. Hematoxylin and eosin (H&E) staining of muscle fibres in animals receiving transgenic C2C12 cells. Increased fibre cross-sectional area (CSA) is observed in treated groups compared to controls.

with those of the control group, but their liver activity did not change. They concluded that IGF1 has a local paracrine effect on muscle tissue with endocrine and autocrine effects on liver activity [33]. Our results for body and leg weights, shown in Figs. 11 & 12, were consistent with the results of their study.

In a study by Liu et al. (2006), a lentiviral vector encoding the GH1 gene was used to investigate the long-term and continuous expression of the GH1 gene in mouse myoblast cells in vitro. Their results revealed that after eight weeks, GH1 gene expression was stable [34].

In this study, we observed sustained in vitro expression of GH1 in C2C12 cells, as well as GH1 expression in rat muscle cells infected with recombinant virus; however, the in vivo expression of this gene did not affect the body weight or muscle weight of the treated rats. This may be due to a variety of factors, including the long half-life of this gene. Therefore, it is not able to reach the right recipients or perform [35]. In this study, the body weight and muscle weight of rats treated with recombinant viruses containing both GH1 and IGF1 increased, which was in accordance with the results of studies in rats that received the genes alone and other studies. Above, it can be concluded that this weight gain is due to the local presence of IGF1, which can stimulate growth hormone activity during growth and increase metabolism.

The results of fluorescence microscopy-based histological studies, as shown in Fig. 13, revealed that GFP expression was due to the presence of transduced cells. These cells contained GH1 and IGF1 as well as proof

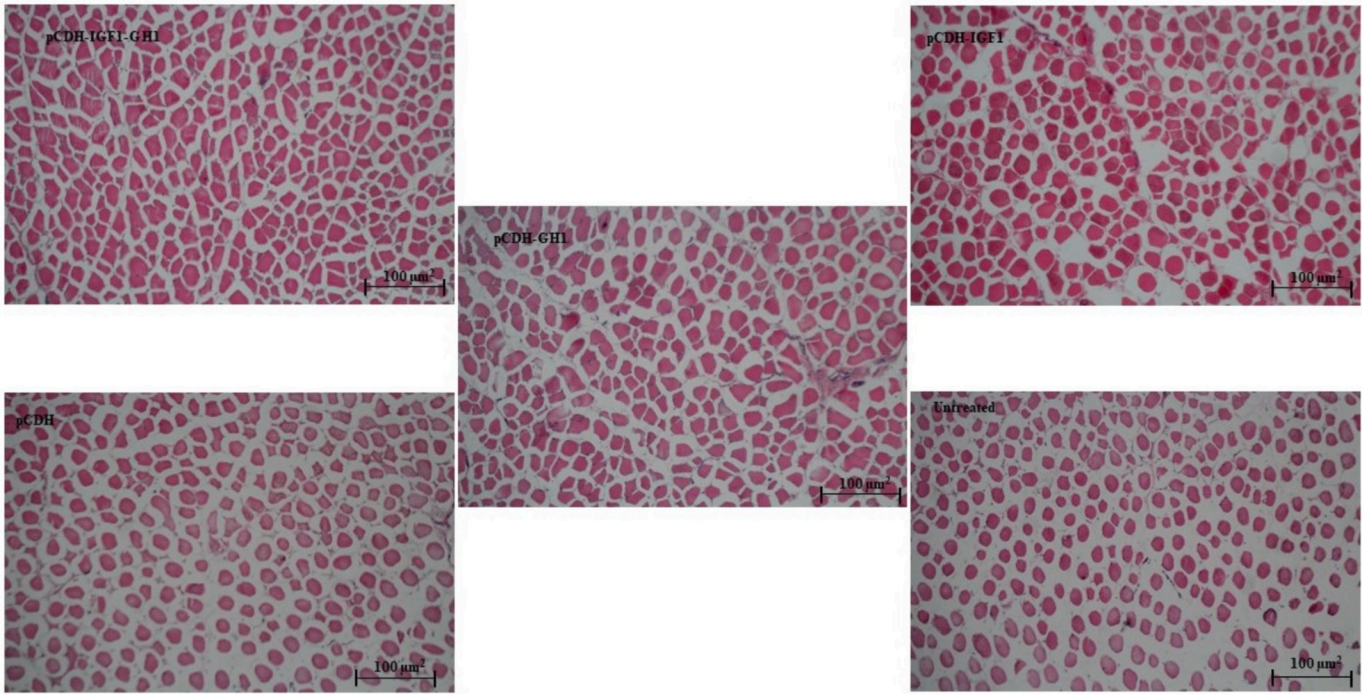

Fig. 15. Hematoxylin and eosin (H&E) staining of muscle fibres from animals receiving lentivirus injections. Top left: pCDH-IGF1-GH1; bottom left: pCDH; centre: pCDH-GH1; top right: pCDH-IGF1; bottom right: untreated control.

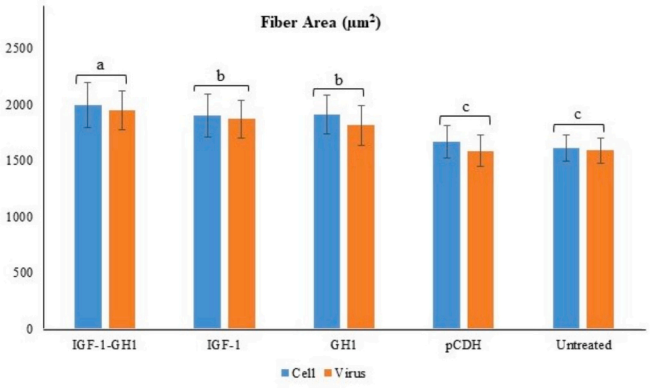

Fig. 16. Comparison of muscle fibre size after the injection of viruses and transduced cells. (For all groups with the same letter, the difference between the means is not statistically significant. If two groups have different letters, they are significantly different (p-values<0.05).

for the integration of the recombinant virus into the tissue. This explains the presence of GH1 and IGF1 and their constant expression in muscle tissue. Consistent with our results, numerous reports that the continuous expression of these genes results in cell proliferation and increased muscle size [34,36].

Owing to the characteristics of the IGF1 gene as a muscle growth factor, it is closely linked to growth hormone. Therefore, histological studies based on hematoxylin–eosin (H&E) staining were performed to identify the effects of the GH1 and IGF1 genes on changes in muscle fibre size. As shown in Figs. 14 & 15, the size of muscle fibres in the muscle tissues of rats receiving recombinant virus containing the IGF1 gene was greater than that in the other groups; these results are in line with the results obtained by Barton et al. (2002) [37].

Limitations of this study was …

6. Conclusion

In this study, it is demonstrated that the injection of lentiviral vectors expressing GH and IGF1 into the muscle of male rats led to significant increases in body and muscle mass. While these findings suggest that lentiviral-mediated gene delivery can promote muscle growth, further studies are needed to confirm the expression of GH and IGF1 at the RNA and protein levels in the treated muscles. Future work should also explore the mechanisms underlying these effects, including the potential systemic release of GH and IGF1 and their impact on overall growth.

Funding statement

This study was supported by Ferdowsi University of Mashhad, grant number: 3/29769.

Ethics approval statement

All animal procedures were approved by Ferdowsi University of Mashhad's ethical committee, permit number: 3/29769.

Patient consent statement

Not applicable.

Permission to reproduce material from other sources

Not applicable.

Clinical trial registration

Not applicable.

CRediT authorship contribution statement

Zahra Roudbari: Writing – original draft, Visualization, Methodology, Formal analysis. **Akram Alizadeh:** Methodology, Data curation.

Mohammadreza Nassiri: Project administration, Funding acquisition, Conceptualization. **Ali Fallah:** Resources, Data curation. **Ali Javadmanesh:** Writing – review & editing, Validation, Investigation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mohammadreza Nassiri reports financial support and equipment, drugs, or supplies were provided by Ferdowsi University of Mashhad. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability statement

The data that supports the findings of this study are available in figures and tables of this article.

Acknowledgements

This study was supported by Ferdowsi University of Mashhad, grant number: 3/29769.

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