

Development of a model to study *Adamts3* gene in myogenic cells

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ARTICLE INFO	ABSTRACT
Article history: Received 14.11.2025 Received in revised form 28.01.2026 Accepted 16.02.2026	The research involved culturing C2C12 cells and transfecting them with a pX459 plasmid containing double-guide RNA (dgRNA), Cas9 protein, and a puromycin resistance gene (puroR) by using JetOPTIMUS transfection reagent. Post-transfection, cells were treated with puromycin, an antibiotic that inhibits protein synthesis, allowing only cells with the integrated resistance gene to survive. Following puromycin selection, cells with successful modifications were clonally expanded and genotyped, revealing a transfection efficiency of 12.2%, with 77% of expanded clones showing wild-type genotypes. These findings suggest the need to refine the gene-editing strategy, potentially by using single-guide RNAs (sgRNAs) and optimizing transfection conditions. Although the targeted exon 2 deletion was not achieved, this model provides insights into gene-editing approaches and the involvement of <i>Adamts3</i> in myogenesis.
Keywords CRISPR/Cas9, <i>Adamts3</i> , Myogenesis, Puromycin selection, Double-guide RNA (dgRNA), NHEJ DNA repair	

Introduction

Skeletal muscle tissue is important for vital functions such as locomotion and respiration. Furthermore, it helps maintain glucose homeostasis and thermoregulation, stores amino acids during critical illnesses, and protects bones and internal organs. Loss of muscle mass due to aging or disease can impair these functions, leading to frailty, increased risk of falls and fractures, and loss of independence [1]. There are significant individual differences in muscle mass even before aging begins to reduce it. Studies indicate that 30–60% of these differences are due to genetic variability [5]. Reverse genetics studies have revealed several essential genes involved in myogenesis and growth [9]. Nevertheless, it is uncertain whether natural variants in these genes demonstrate differences in muscle mass and function. Recent genome-wide association studies (GWAS) reveal that the majority of critical genes regulating myogenesis and muscle growth lie beyond the approximately 180 genomic areas linked to muscle mass in humans [6].

This project focuses on the *Adamts3* gene because of its undiscovered significance in muscle tissue, particularly in myogenic cells. *Adamts3* belongs to the ADAMTS family (A Disintegrin and Metalloproteinase with Thrombospondin Motifs), which is involved in remodeling the extracellular matrix (ECM), a process vital for muscle development and repair.

Moreover, recent *in silico* studies have demonstrated the significance of particular single nucleotide polymorphisms (SNPs) in the *Adamts3* [10]. Cells have the ability to proliferate, grow, and differentiate into contractile myotubes [4]. This study utilizes CRISPR/Cas9 technology in C2C12 cells to develop a reliable model to elucidate the functions of *Adamts3* in muscle development and pathology [7]. However, this repair mechanism is error-prone and can lead to deletions and functional disruptions in the *Adamts3* gene [8].

Materials and methods

To investigate the role of the *Adamts3* gene in myogenic lineage, exon 2 was specifically targeted for excision in C2C12 cells. Subsequent to this targeted modification, clones of C2C12 cells were developed and genotyped to test the accuracy of the genetic modification.

Cell culture. C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS, Gibco™) and 2% glutamine (Gibco™). They were maintained at 37°C and 5% CO₂ in a humidified incubator and passaged regularly when they had 50-70% confluence [4].

Transfection. Cell transfection enables the introduction of genetic materials within the pX459 plasmid (see figure 1) into low-passage C2C12 cells by using JetOPTIMUS[®] (Polyplus-transfection[®] SA, New York, NY) transfection reagent [8]. The dgRNA consists of a CRISPR RNA (crRNA) and a trans-activating CRISPR RNA (tracrRNA), which together form a complex that guides the Cas9 protein to the specific genomic target site, facilitating precise DNA cleavage [12]. JetOptimus[®] was chosen over other transfection reagents due to its distinct formulation that enhances the delivery of the CRISPR/Cas9 plasmid into mammalian cells, including the notoriously difficult-to-transfect myoblast cells such as C2C12. The formulation of JetOptimus[®] is specifically designed to protect the DNA during the process, enhance cellular uptake, and minimize cytotoxicity [4].

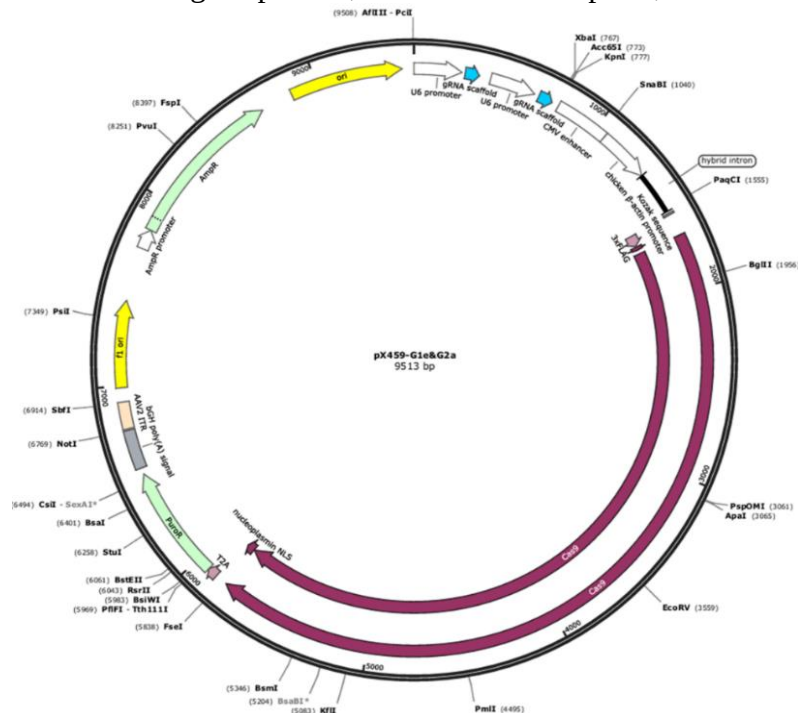


Figure 1. Plasmid map of X459-G1e&G2a (9513 bp)

The pX459-G1e&G2a plasmid was engineered by Ms. Sonja Rauber. It is a 9513-base pair circular DNA construct designed for precise gene editing applications. This plasmid includes several essential components: dgRNA, Cas9 protein, and puroR.

The transfection procedure was done for 2 days following a modified protocol described by Taye and colleagues [11]. On the first day, a cell suspension of 48,000 C2C12 cells in 1 mL of growth medium (GM) was prepared, with 500 μ L of the suspension added to two wells of a 24-well plate (density of 12.5k cells/cm²). One well was labeled as transfected and the other as a control, and both were incubated overnight. The following day, both of the wells were replaced with Growth Medium. The transfection reagent was brought to room temperature and briefly vortexed. A 1:1.5 dilution of 0.5 μ g plasmid DNA in 50 μ L of JetOPTIMUS buffer was prepared, vortexed for 1 second, and briefly spun down. After a 4-hour incubation, the medium was replaced with fresh

growth medium. Transfected myoblasts were cultured for 24 hours to allow for the expression of the transfected plasmid DNA [4].

Puromycin Selection. In this step, the medium in which the cells were growing was replaced with a fresh GM. The cells were then exposed to the antibiotic at a concentration of 5 µg/mL for 5 days according to the protocol described by Taye and colleagues [11].

Clonal Expansion. Following puromycin selection, the surviving cells were expanded for a week and subjected to clonal selection in accordance with the protocol described by Bauer and colleagues [2]. Initially, the transfected well was refreshed with new GM while the control well was discarded as all of them died. During the following 2-3 weeks, well-plates were screened with an inverted microscope, and 57 wells contained colonies of single-cell origin.

Genotyping. The final step in the process was genotyping the expanded clones to confirm the deletion. First of all, GM was removed and the cells were washed with PBS, trypsinized, and 20% of the cells were seeded in a 24-well plate for further culture, while the remaining 80% were transferred to an Eppendorf tube and genotyped.

PCR amplification of the CRISPR targeted region. Using the NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>), a pair of primers was designed to amplify a region spanning the gene deletion site [3]. Sequences of primers used are presented in Table 1 and target region for *Adamts3* gene is shown in Fig 2.

Table 1.

Sequence of primer (with amplicon size) used to amplify CRISPR target region of Adamts3 gene.

Gene Targeted	Direction and Name	Primer Sequences (5' to 3')	Amplicon size
Adamts3	Forward (A3-F2)	CCAAAGAGGCCATTGAGGGT	555 bp
	Reverse (A3-R2)	CAGCTTGCACCCAGTCAGTA	

Guide1 GAGCCTTCTAGGAACAACCG
 Guide2 GATAAGAGTGACATTTGAGA (note: complementary strand)

ggcatttctctagttgattcttaggattcaggccacttctcacctggtgaaatgtagactac
 gccccagcccccccccccttctgattcgtgtgacttagtattggagttttccaaagag
 gccattgaggggcactattttactctcttcacagataactaaaattaaagctggccaacagt
 cctctctcactagtcataagaattggctcatgttctctgtcacaaagaccccttctaggaacaa
 ccgggggtcaggcttcaggcagcaattccagtcctctgggtctcctgattagccagtcacccc
 aaagcactgatggtagaaataaccaagcagcgagataagatgaaaccagtaaccgtttctt
 ttcttctgctccccaccctcctttccagGCTGGTACTGACGAAATGGTGCAAATAGgtaagc
 ctcttctctcaaatgtcactcttatctagctgtgagctgagaacaaagttaaggccaagcaa
 aagaactgtttctgaaagtcttctaatgagtggttaaaataatggatagtccttgaatcatt
 tctactcttctgctttccgtgggtgggtcgatgttctcctcgggctaagttatagtgcatca
 atagccttccacttctgagcatcaatttatTactgactgggtgcaagctgctgctatgcc
 tgggagagttacagtgggtcggaacaccagagatgtataaagtttagaccctcaacttgtgt
 aacataaggagaaacaaattagttataactgaagacggtgtcaggatgctagagataaagg
 gaaaaagatgaagagaaaattc

Figure 2. Sequence of *Adamts3* gene derived from the muscle of mouse. The PCR amplified region encompassing the CRISPR target in exon 2 is indicated in red, guide RNA sequences are highlighted in green and the positions of the primer (F2 and R2) used for PCR amplification are shown in pink (see amplicon sizes in Table 1).

Image Analysis. The acquired images of transfected and puromycin-selected cells were analyzed using ImageJ software. These measurements were used to set the scales to 200 µm and 400 µm correspondingly. Next, a square with dimensions 500 µm by 500 µm was drawn in the center of the image using the rectangle tool. The images were analyzed to determine the number of cells per 250,000 µm².

Results

To establish an effective CRISPR/Cas9 system, the pX459 plasmid containing a dgRNA, a Cas9 nuclease expression cassette, and a puromycin N-acetyltransferase was introduced for the selection of transfected cells (Figure 3).

Transfection and puromycin selection results: Initially, low-passage C2C12 cells were transfected, with one well serving as the control. As a result, 82 transfected and 21 control cells were counted (Figure 3A). Furthermore, selection of puromycin-resistant C2C12 cells was achieved within 9 days post-transfection due to the efficient elimination of non-resistant cells (Figure 3B). Typically, more than 80% of the cells detached from the cell culture dish and were eliminated during regular maintenance. The transfection efficiency is 12.2%, as indicated by the survival of 10 out of 82 cells after puromycin selection (Figure 3C).

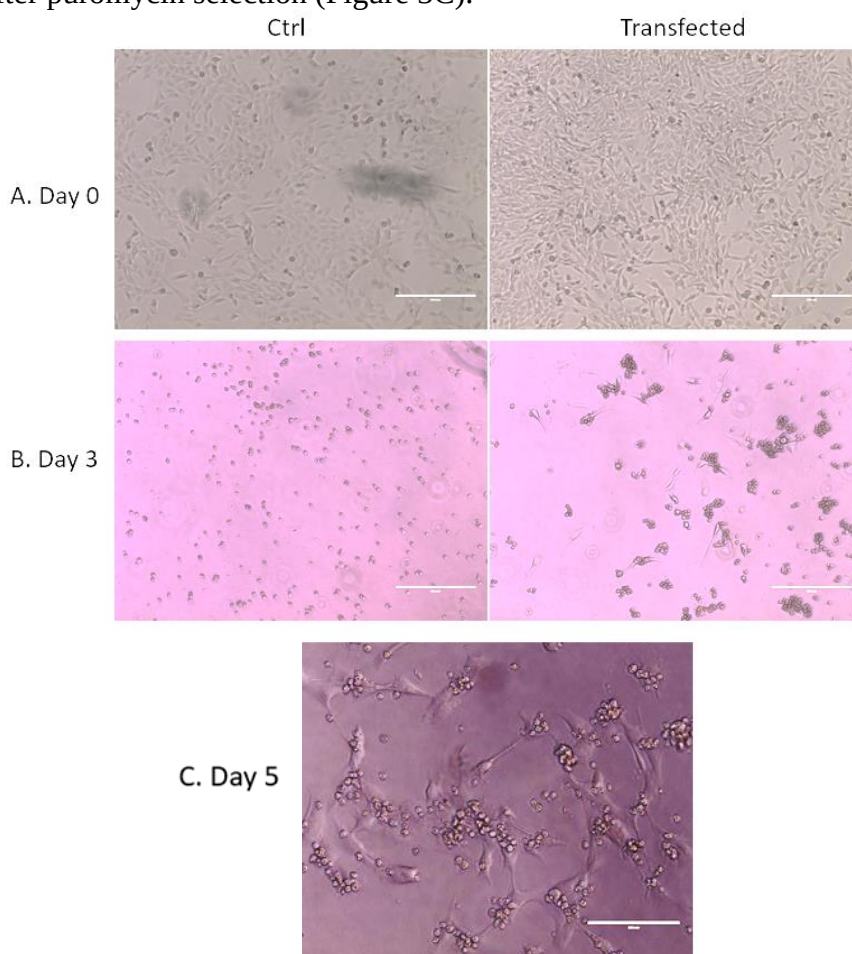


Figure 3. The efficiency of transfection and subsequent puromycin selection in C2C12 cells.

(A) The panels demonstrate images of control and transfected cells cultured in complete DMEM immediately after transfection, with pX459 plasmid showing similar cell density. Scale bars = 400 μ m.

(B) The panels demonstrate the effect of puromycin selection by day 3, showing that only transfected cells with the puromycin resistance gene survive, while control cells have died. Scale bars = 400 μ m.

(C) The panel demonstrates the continued viability of transfected cells by day 5, indicating the successful selection and transfection. Scale bars = 200 μ m.

Clonal selection and genotyping results: Surviving cells were expanded as a total of 600 C2C12 cells were seeded throughout nine 96-well culture plates. The growth of the cells was

monitored over a period of 2-3 weeks. Out of all the colonies observed during this time, a total of 57 colonies were chosen. It accounts for 9.5% of the seeded cells. Moreover, out of these 57 colonies, the majority (96.5%) were made up of single cells, while a small portion (0.8%) comprised of two colonies (see Figure 4).

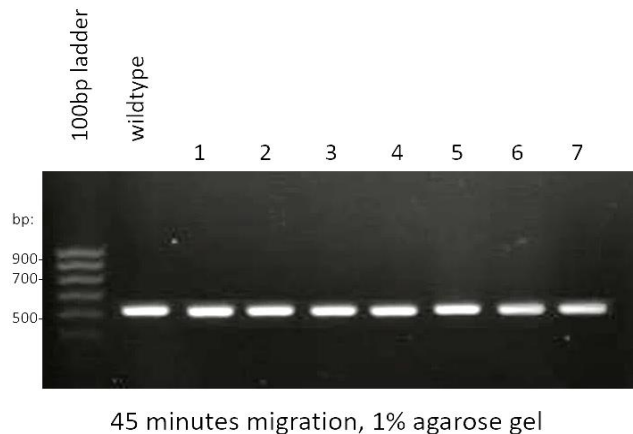


Figure 4. Screening of C2C12 clones generated from CRISPR-Cas9 plasmid pX459 to detect potential deletion of exon 2. The CRISPR-targeted locus was PCR amplified from genomic DNA using 555 bp amplicon size, and PCR products were size-separated by electrophoresis on a 1% agarose gel for 45 minutes.

Discussion

The study aimed to develop a model to study the role of *Adamts3* in myogenic tissue development and growth by generating C2C12 cell clones with a targeted deletion of exon 2 using CRISPR/Cas9 technology. The outcomes of transfection, puromycin selection, clonal expansion, and genotyping indicated that all of these clones ended up with wild-type genotypes, suggesting potential directions for further research.

These findings suggest that utilizing sgRNAs instead of dgRNAs may provide effective consequences in this research. This approach ensures that both guides are capable of inducing DSBs independently, increasing the likelihood of successful exon excision when used together, as the research performed by Ran et al. (2013) demonstrated that this approach is highly effective in guiding Cas9 to specific genomic loci [8].

Moreover, optimizing the transfection conditions may lead to achieving maximal efficiency without inducing cytotoxicity. Studies by Cocchiariaro et al. (2014) have demonstrated that optimizing these parameters is essential for achieving high transfection efficiency while minimizing cell death. Reagents can be designed specifically for C2C12 cells with the higher concentration of reagents, considering the toxicity effect and increasing efficiency. Viafect[™] and Fugene[®] HD reagents show higher efficiency when reducing cell confluency, while higher cell density was required to limit cytotoxicity conferred by Avalanche[®] [4].

To conclude, this research model suggests potential outcomes for investigating muscle differentiation and growth, whereas the role of the *Adamts3* gene in C2C12 cells still needs further study. By considering the use of sgRNAs, optimization of transfection conditions, and contextualizing these findings within the broader field of myogenic research, this model demonstrates a comprehensive approach for identifying the role of this gene in muscle development and growth.

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Xülasə

Miogen hüceyrələrdə *Adamts3* geninin tədqiqi üçün modelin tətbiqi Qasimova G.E.

Açar sözlər: CRISPR/Cas9, *Adamts3*, Miyogenez, Puromisin seleksiyası, İki-bələdçi RNT (dgRNA), NHEJ DNT bərpası

Bu tədqiqatın məqsədi *Adamts3* geninin funksional rolunu müəyyənləşdirmək üçün Clustered Regularly Interspaced Short Palindromic Repeats-associated protein 9 nuclease (CRISPR/Cas9) texnologiyasından istifadə etməklə genin 2-ci ekzonunu hədəfləyən (*Adamts3*-ΔE2) modelin yaradılması olmuşdur. Bu yanaşma, genin funksional aktivliyini dayandırmaq məqsədilə mutasiyaların induksiya edilməsinə əsaslanmışdır.

Tədqiqat çərçivəsində C2C12 hüceyrə xətti kultivasiya edilmiş və pX459 plazmidi vasitəsilə transfeksiya olunmuşdur. Sözündən plazmid iki-bələdçi RNT (dgRNA), Cas9 proteini və puromisinə davamlılıq geni (puroR) daşıyır. Həmçinin plazmid JetOPTIMUS transfeksiya reagentindən istifadə olunaraq hüceyrələrə daxil edilmişdir. Transfeksiyadan sonra hüceyrələr puromisin antibiotiki ilə seleksiya olunmuşdur. Puromisin zülal sintezini blokladığından yalnız davamlılıq genini daşıyan hüceyrələr sağ qala bilmişdir. Puromisin seleksiyasından sonra uğurla modifikasiya olunmuş hüceyrələr klonlaşdırılaraq çoxaldılmış və genotipləndirilmişdir. Nəticələrə əsasən, transfeksiya effektivliyi 12,2% olmuş, klonlaşdırılmış hüceyrələrin isə 77%-nin dəyişməmiş (wild-type) genotipə malik olduğu müəyyən edilmişdir. Əldə edilmiş nəticələr gen-redaktə strategiyasının təkmilləşdirilməsinin zəruriliyini təsdiqləyir — xüsusilə tək-bələdçi RNT (sgRNA) tətbiqinin və transfeksiya prosesinin optimallaşdırılmasının vacibliyini vurğulayır.

Резюме

Разработка модели для изучения гена *Adamts3* в миогенных клетках

Гасимова Г.Э.

Ключевые слова: CRISPR/Cas9, *Adamts3*, миогенез, отбор пурамицином, двойная направляющая РНК (dgRNA), репарация ДНК по механизму NHEJ.

В исследовании осуществлялось культивирование клеток C2C12 и их трансфекция плазмидой pX459, содержащей двойную направляющую РНК (dgRNA), белок Cas9 и ген устойчивости к пурамицину (puroR), с использованием трансфекционного реагента JetOPTIMUS.

После трансфекции клетки обрабатывали пурамицином — антибиотиком, который ингибирует синтез белка, что позволяло выжить только клеткам с интегрированным геном устойчивости. После отбора пурамицином клетки с успешными модификациями подвергались клональному расширению и генотипированию, что показало эффективность трансфекции 12,2%, причём 77% расширенных клонов имели генотип дикого типа. Эти данные указывают на необходимость уточнения стратегии редактирования генов, возможно, с использованием однонаправляющих РНК (sgRNA) и оптимизацией условий трансфекции. Несмотря на то что удаление целевого экзона 2 не было достигнуто, эта модель предоставляет представление о подходах к редактированию генов и роли *Adamts3* в миогенезе.