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A 3D culture method based on interpenetrating hydrogels induced by cells into skeletal muscle tissue in vitro

Guobiao Wang

Xidian University, 266 Xinglong Section, Xifeng Road, Chang 'an District, Xi 'an City, Shaanxi Province, 710126, China

godvvv668@163.com

Abstract. The combination of muscle cells and soft robotics technology has led to the rapid development of biohybrid robots in recent years. Muscle-driven systems had advantages such as joint flexibility, self-repair, and multi-signal perception, demonstrating unique advantages as an effective technological solution for robot drive systems. Current research has many shortcomings in material properties and muscle stability in 3D muscle tissue cultured in vitro, making it difficult to successfully apply the technology. In this study, we developed a new type of interpenetrating hydrogel network that, combined with 3D cell culture and tissue-induced culture, enabled 3D muscle tissue culture in a hydrogel environment and induced differentiation in muscle tissue. The results of this research provided a foundation for future studies on in vitro muscle tissue culture and the implementation of robots in this field.

Keywords: muscle-driven systems, interpenetrating hydrogel network, skeletal muscle tissue, horse serum.

1. Introduction

In recent years, the continuous development of technologies such as 3D printing, cell engineering, and biomaterials has brought biohybrid robots that combine biological cells and soft robots into our view [1]. Soft robots with muscle-driven systems were expected to combine the advantages of traditional robots' rigid structures and biological tissue's flexible driving methods [3], such as joint flexibility [3], self-repair [4], and multi-signal control [5], enabling robots to perform more complex actions and meet diverse human needs [6].

Skeletal muscle tissue was susceptible to drying and damage in the air, making it difficult to start in a non-liquid environment [9]. Therefore, a biohybrid robot was proposed, in which skeletal muscle tissue was encapsulated in a collagen structure to maintain the required humidity conditions when operating in the air. Despite being encapsulated in a collagen structure, the biohybrid robot could still move in the air by contracting skeletal muscle tissue, which maintained high cell viability and contractility. However, muscle tissue grown outside the body lacks real nutritional exchange, leading to decreased nutritional performance. The use of extracellular matrix (ECM) and collagen-cultured muscles was time-consuming [10], and the resulting organ heterogeneity was difficult to control due to culture material differences. [11]

Therefore, using a new type of hydrogel to construct a gel network to achieve 3D culture of muscle tissue has become a focus of research. The use of hydrogel to form an interpenetrating network could

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effectively utilize the advantages of different hydrogels for tissue culture. In this study, interpenetrating hydrogel technology and 3D skeletal muscle culture technology were combined to produce in vitro skeletal muscle tissue in the laboratory. Under 3D culture conditions, induced differentiation in the skeletal muscle tissue produced muscle tubes, completing the 3D culture of skeletal muscle tissue in vitro.

2. Methods

2.1. In vitro cell culture

C2C12 mouse skeletal myoblasts (ATCC) Cells were maintained in growth medium (GM) consisting of DMEM with L-glutamine and sodium pyruvate (Corning Cellgro) supplemented with 10% (vol/vol) FBS (Lonza), 1% (vol/vol) L-glutamine (Cellgro Mediatech), and 1% (vol/vol) penicillin-streptomycin (Cellgro Mediatech)[12-13].

2.2. Gtn–HPA gel formation and cell seeding

In this study, a 4% Gtn–HPA solution was prepared first by dissolving the lyophilized Gtn–HPA conjugate in phosphate-buffered saline (PBS) at 37 °C and then mixing with cell suspension or DMEM (Life Technologies, USA) to reach a final concentration of 2%. The gel formulation used was crosslinked by 0.1 U/mL HRP (Wako Chemicals, USA) and 1.2 mM H_2O_2 (Sigma-Aldrich, USA)[14-15].

2.3. Immunofluorescence

DAPI/phalloidin images were obtained to examine the nuclei and F-actin of the cells. Cells were fixed with 3.7% paraformaldehyde overnight at room temperature. After permeabilization with 0.2% Triton X-100 for 10 min, the cells were stained with diamidino-2-phenylindole (DAPI) and phalloidin (15 U mL-1)[16].

2.4. Image acquisition

Images were captured using confocal microscopy (LSM 700, Zeiss, Germany).

3. Results

3.1. Using an interpenetrating hydrogel l network for 3D cultivation of skeletal muscle cells

To obtain three-dimensional muscle tissue in a 3D culture environment, we used hydrogels to perform skeletal muscle cell culture. In the experimental procedure, 15 mg/mL agarose solution was firstly added to the bottom of the dish by adding 50 μL to form a gel, and then different concentrations of Gtn+HPA (I) were added. Next, serum-free cell-containing medium was added to 200 μl in each well and mediated to mix well (II), then HRP was added to the gel (III), and finally, H_2O_2 was added to the gel and put into the incubator for cross-linking (1-4 h) (IV) (Figure 1A). Under different concentrations of hydrogel culture environment, a large number of cell spheres were found to be present after 40 h of cultivation (Figure 1B).

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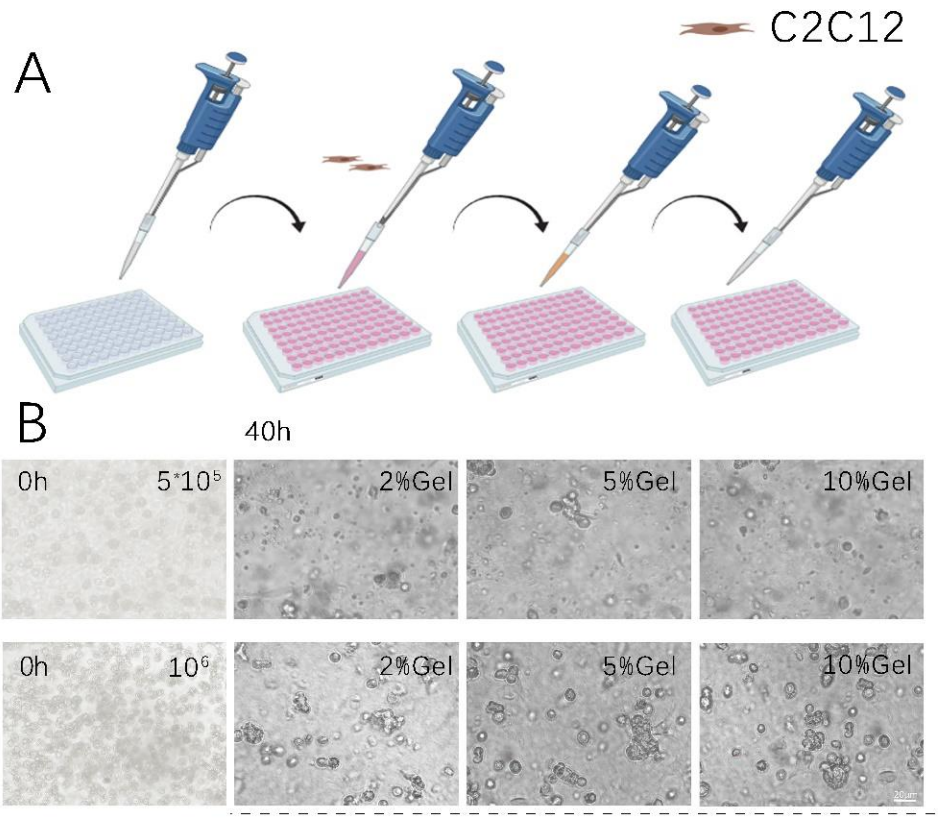

Figure 1. Using hydrogels for skeletal muscle cell culture (A) Experimental procedure for cell culture In the experimental procedure, 15 mg/mL agarose solution was firstly added to the bottom of the dish by adding 50 μL to form a gel, and then different concentrations of Gtn+HPA (I) were added. Next, serum-free cell-containing medium was added to 200 μl in each well and mediated to mix well (II), then HRP was added to the gel (III), and finally, H_2O_2 was added to the gel and put into the incubator for cross-linking (1-4 h) (IV). (B) Microscopic morphology of $5*10^5$ and 10^6 cells/mL at different hydrogel concentrations from 0 to 40 h. (Scale bars = 20 μm).

3.2. Obtaining 3D muscle tissue

In order to obtain visible muscle tissue, muscle cell sphere tissue was formed after 14 days of long-term 3D culture, with a large amount of muscle cell sphere tissue present at a cell concentration of 106 cells/ml and a 2% hydrogel culture environment. Regardless of the different cell concentrations, muscle cell spheres (150-200 μm) were present. And the lower the hydrogel concentration, the larger the cell spheres (Figure 2).

To differentiate 3D-cultured skeletal muscle cell spheres into muscle and obtain mature muscle tissue, we used horse serum (2% horse serum) for muscle cell sphere induction and found that muscle masses appeared extended and elongated after 14 days of culture + 3 days of induction, and different cell spheres were contacted to fuse into larger tissue masses (Figure 3).

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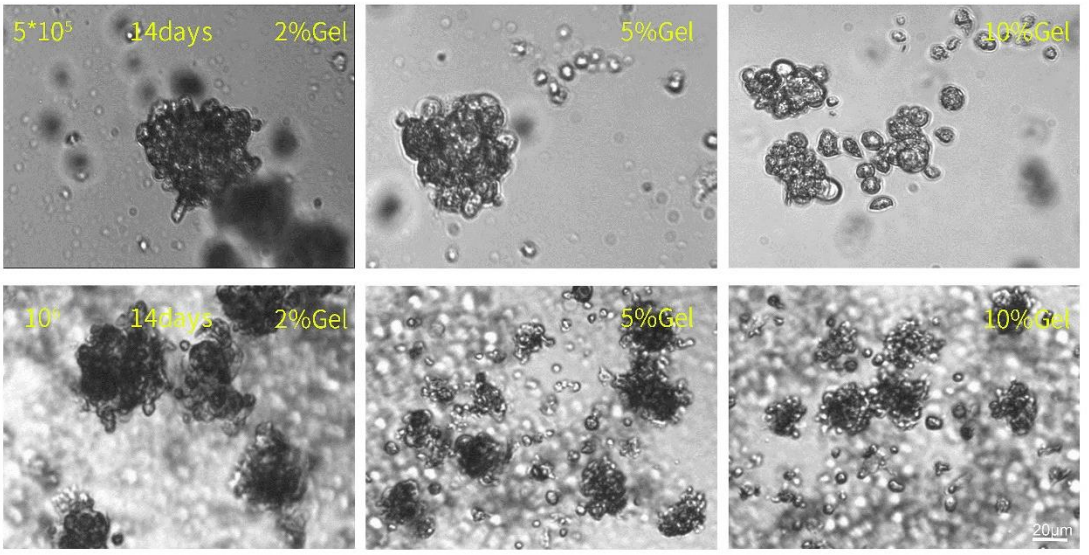

Figure 2. 3D microscopic display of $5*10^5$ and 10^6 cells/mL at different hydrogel concentrations 3D cultured muscle cell spheres after 14 days of 3D culture (Scale bars = 20 μm).

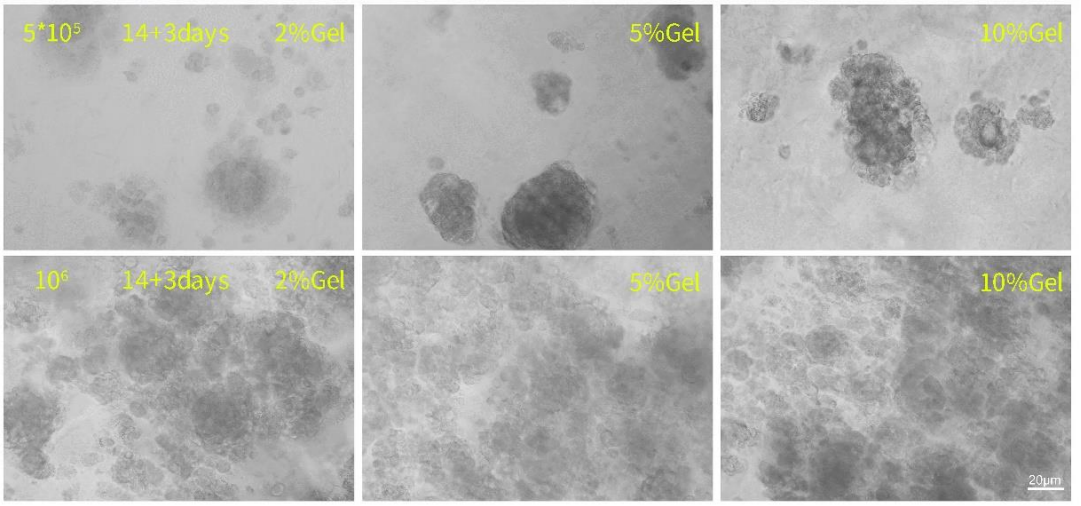

Figure 3. 3D microscopic display of $5*10^5$ and 10^6 cells/mL at different hydrogel concentrations 3D-cultured skeletal muscle cell spheres were induced using horse serum (2%) for 3 days. (Scale bars = 20 μm).

4. Myogenesis of muscle tissue

In order to research the differences in cell morphology and characteristic actin morphology before and after muscle cell induction, we stained F-actin using the immunofluorescence technique. Under 2D culture conditions without induction, F-actin fibers were found to be more disorganized (Figure 4A), and after induction with 2% horse serum for 6 days, the fibers were more uniform and myotubes were clearly visible (Figure 4B).

To characterize the myotubular fibers I obtained, these myotubular fibers were formed by differentiation after induction of 3D skeletal muscle masses. Using immunofluorescence to stain uninduced 3D cultured skeletal muscle cell masses, it was observed that there was no obvious myotube formation in skeletal muscle tissues (Figures 5A, B, and C). Under 3D culture conditions, there was obvious myotube production after 14 days of induction using horse serum, and it could be found that

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the myotube direction was the same as the direction of induction by serum addition (Figures 5D, F, and E).

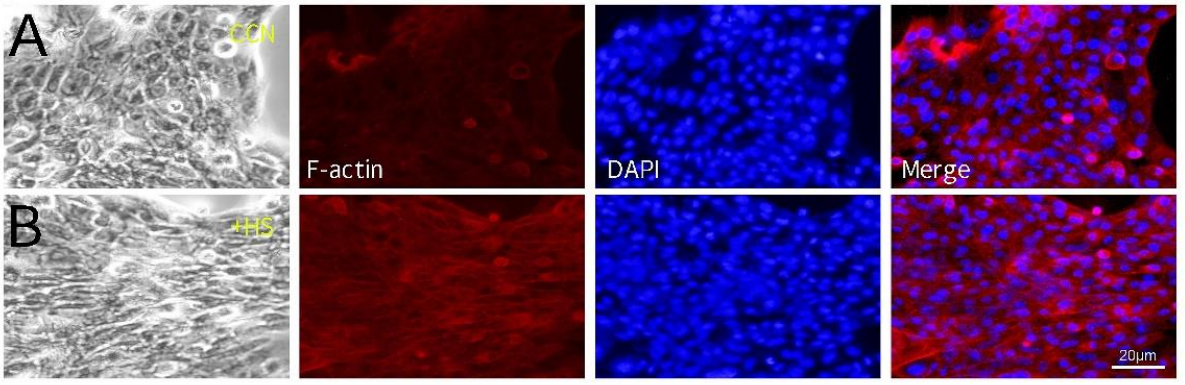

Figure 4. Immunofluorescence of 2D skeletal muscle cells In a 2D Petri dish, immunofluorescence staining was performed, and F-actin was red, DAPI was blue, and the merge (A). In a 2D Petri dish, after induction by adding horse serum (2%), immunofluorescence staining was performed; F-actin is red, DAPI is blue, and the merge (B). (Scale bars in A and B = 20 μm).

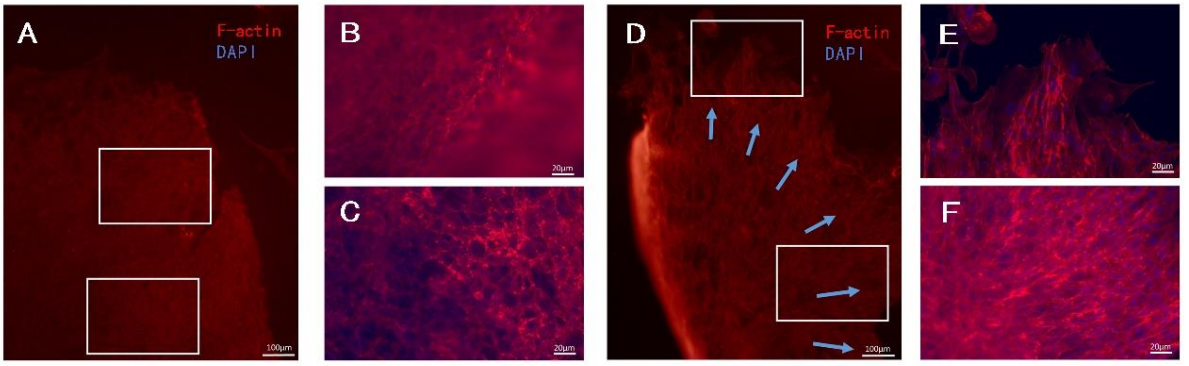

Figure 5. Immunofluorescence of 3D skeletal muscle Using immunofluorescence, muscle tissue in culture was stained, and F-actin was red and DAPI was blue. (A). (Scale bars in A = 100 μm) (B) and (C) indicate the magnified image in (A) of the white box. (Scale bars in B and C = 20 μm) Using immunofluorescence, the cultured muscle tissue is stained, and F-actin is red and DAPI is blue. The arrow indicates the orientation of the myotubular fibers (D). (Scale bars in A = 100 μm) (E) and (F) indicate the magnified image in (D) of the white box. (Scale bars in E and F = 20 μm).

5. Conclusion

By using a novel 3D culture scaffold constructed from interpenetrating hydrogels, we achieved 3D culture of muscle cells in tissue masses and induced myogenesis in a 3D environment. The success of in vitro culture of skeletal muscle cells into muscle tissue organoids provides an alternative tissue foundation for future soft robots and has the potential to become a drug detection platform.

References

[1] Duffy RM, Feinberg AW. Engineered skeletal muscle tissue for soft robotics: fabrication strategies, current applications, and future challenges. Wiley Interdiscip Rev Nanomed Nanobiotechnol. 2014;6(2):178-195. doi:10.1002/wnan.1254

[2] Gao L, Akhtar MU, Yang F, et al. Recent progress in engineering functional biohybrid robots actuated by living cells. Acta Biomater. 2021;121:29-40. doi:10.1016/j.actbio.2020.12.002

[3] Li Z, Seo Y, Aydin O, et al. Biohybrid valveless pump-bot powered by engineered skeletal muscle. Proc Natl Acad Sci U S A. 2019;116(5):1543-1548. doi:10.1073/pnas.1817682116
[4] Dumont NA, Bentzinger CF, Sincennes MC, Rudnicki MA. Satellite Cells and Skeletal Muscle Regeneration. Compr Physiol. 2015;5(3):1027-1059. doi:10.1002/cphy.c140068
[5] CVETKOVIC C , RAMAN R , CHAN V , et al. Three-dimensionally printed biological machines powered by skeletal muscle[J]. Proc. Natl. Acad. Sci. U. S. A, 2014, 111(28): 10125-10130
[6] Aydin O, Zhang X, Nuethong S, et al. Neuromuscular actuation of biohybrid motile bots. Proc Natl Acad Sci U S A. 2019;116(40):19841-19847. doi:10.1073/pnas.1907051116
[7] Cvetkovic C, Raman R, Chan V, et al. Three-dimensionally printed biological machines powered by skeletal muscle. Proc Natl Acad Sci U S A. 2014;111(28):10125-10130. doi:10.1073/pnas.1401577111
[8] Ricotti L, Menciassi A. Bio-hybrid muscle cell-based actuators. Biomed Microdevices. 2012;14(6):987-998. doi:10.1007/s10544-012-9697-9.
[9] Yuya Morimoto, Hiroaki Onoe, and Shoji Takeuchi, "Biohybrid robot with skeletal muscle tissue covered with a collagen structure for moving in air", APL Bioengineering 4, 026101 (2020) https://doi.org/10.1063/1.5127204
[10] Cheng KF, Her WY, Liu TS, Chen SC, Liu KM. Primary culture of mouse myoblasts. Gaoxiong Yi Xue Ke Xue Za Zhi. 1995;11(6):306-314.
[11] Jo, B., Morimoto, Y., & Takeuchi, S. (2022). Skeletal muscle-adipose co-cultured tissue fabricated using cell-laden microfibers and a hydrogel sheet. Biotechnology and Bioengineering, 119, 636 - 643. https://doi.org/10.1002/bit.27989
[12] Chakraborty S, Hentrich T, Wetzel F, et al. PPARgamma induced fatty acid oxidation and mitochondrial gene expression are increased by dietary fish oil and fenofibrate in skeletal muscle cells. Biochim Biophys Acta. 2015;1850(2):352-358. doi:10.1016/j.bbagen. 2014.11.019
[13] Pérez-Schindler J, Summermatter S, Salatino S, et al. The corepressor NCoR1 antagonizes PGC-1α and estrogen-related receptor α in the regulation of skeletal muscle function and oxidative metabolism. Mol Cell Biol. 2012;32(3):491-502. doi:10.1128/MCB.06271-11
[14] Chen X, Chen H, Li S, et al. A shear-thinning adhesive hydrogel for endovascular embolization. Sci Adv. 2020;6(36):eabb8096. doi:10.1126/sciadv.abb8096
[15] Wang C, Huang Y, Pan Y, et al. Preparation and characterization of gellan gum-glyceryl monooleate-glycerol monostearate nanoparticles loaded with edaravone for potential use in ischemic stroke. Int J Nanomedicine. 2015;10:6185-6198. doi:10.2147/IJN.S90323.
[16] Zhang Y, Chen W, Feng B, et al. Human embryonic stem cell-derived exosomes promote pressure ulcer healing in aged mice by rejuvenating senescent endothelial cells. Stem Cell Res Ther. 2021;12(1):50. doi:10.1186/s13287-020-02035-3.