

SKELETAL MUSCLE TISSUE IMPROVEMENT BY CO-CULTURE SYSTEM IN GELATIN METHACRYLATE HYDROGEL

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ABSTRACT

In this study, we demonstrate that a co-culture approach between C2C12 myoblasts and pheochromocytoma PC12 neuronal cells allows for better formation of muscle-like tissues than a mono-culture of C2C12 cells. In particular, we used a micromolding technique to fabricate microgrooved patterns of methacrylated gelatin (GelMA) hydrogel, which served as a substrate to culture encapsulated cells in GelMA and to induce myoblasts alignment. GelMA is a semi-natural polymer that is easy to pattern by UV irradiation for making 3D structures in which cells can proliferate. Myoblasts and the resulting myotubes were analyzed in terms of alignment and length, respectively, in the mono-culture and the co-culture systems for both patterned and unpatterned regions.

KEYWORDS

Hydrogel, GelMA, C2C12, PC12, Co-culture

INTRODUCTION

Engineered muscular tissues are far from matching the properties of their natural counterparts. Many attempts to align myoblasts for favoring their differentiation into myotubes have been made in 2D and 3D environments [1, 2]. Co-culture has also been used to improve muscle tissue formation notably by the use of endothelial cells and fibroblasts combined with myoblasts, while primary nerve-myoblasts systems were reserved to study neuromuscular junction [3]. Here, we expand the use of our method to control cells alignment to a co-culture of C2C12 and PC12, and we use neural cells to study their impact on myoblasts differentiation.

EXPERIMENTAL

Mold and stamp fabrication

The SU-8 mold master was fabricated on a silicon wafer by using standard soft-lithography techniques. The design of the mold consisted of 1 cm² SU-8 surface patterned of microgrooves (100 μm width, 50 μm depth) / ridges (50 μm width). PDMS prepolymer mixed with curing reagent (10:1 mass ratio) was poured into the master mold and cured at 70°C for 1.5 h after degassing in a vacuum chamber. The PDMS microstructured layer was then peeled off from the master and silanized 30 min. under vacuum in presence of 30 μl of trichloro (1H, 1H, 2H, 2H tridecafluoro-n-octyl) silane (FOTS) to allow easy release of the PDMS stamp from the GelMA polymer.

GelMA synthesis

Gelatin type A (6g) was dissolved in 60 ml DPBS at 50°C. 20% (v/v) of methacrylate anhydride was mixed and the solution was stirred during 1h. Then 240 ml of warm DPBS (40°C) was added to stop the acrylation reaction. The mixture was then dialyzed for 1 week in 12-14 kDa cutoff dialysis tubes against warm (40°C) milliQ water followed by a freeze drying step during 1 week.

Cells seeding and encapsulation

In this study, we employed a microfabricated PDMS stamp to mold grooves (100 μm width, 50 μm depth) / ridges (50 μm width) micropatterns of 20% GelMA hydrogels on Petri dishes, which were further photopolymerized under UV (7 mW/cm²) for 150 sec. We then loaded the grooves with cells (in monoculture C2C12 or PC12, in co-culture 50% C2C12 + 50% PC12) in a pre-polymer solution of 5% GelMA + 1% photoinitiator in DPBS. This cell laden hydrogel was then photopolymerized under UV for 30 sec (Fig.1).

RESULTS AND DISCUSSION

To obtain a muscular tissue *in vitro* with a substantial contractibility, the C2C12 myoblasts must be aligned toward one direction mimicking *in vivo* situation. Several techniques exist to align cells such as the use of mechanical stimulation, electrical field or surface patterning [4, 5]. In this study, we used the topographical constraints induced on the myoblasts by culturing them in narrow and deep grooves of GelMA micropatterns used as substrate. As shown in Fig. 2, after 5 days of culture in DMEM complemented with 10% fetal bovine serum (FBS) and 1% antibiotics, the myoblasts alignment was

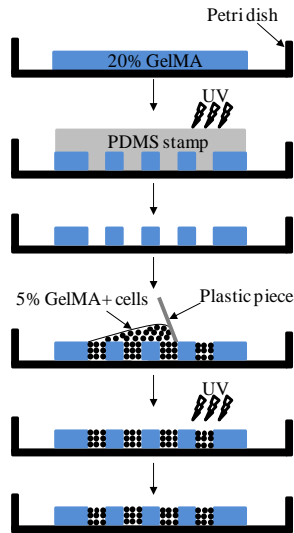


Figure 1: Micromolding process and cell seeding in GelMA.

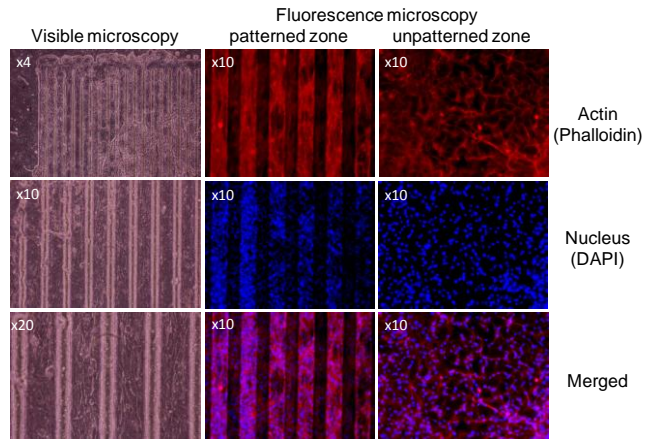


Figure 2: C2C12 alignment in patterned zone (groove width 100 μm , depth 50 μm and interlines gap 50 μm) observed in fluorescence microscopy after actin filaments staining.

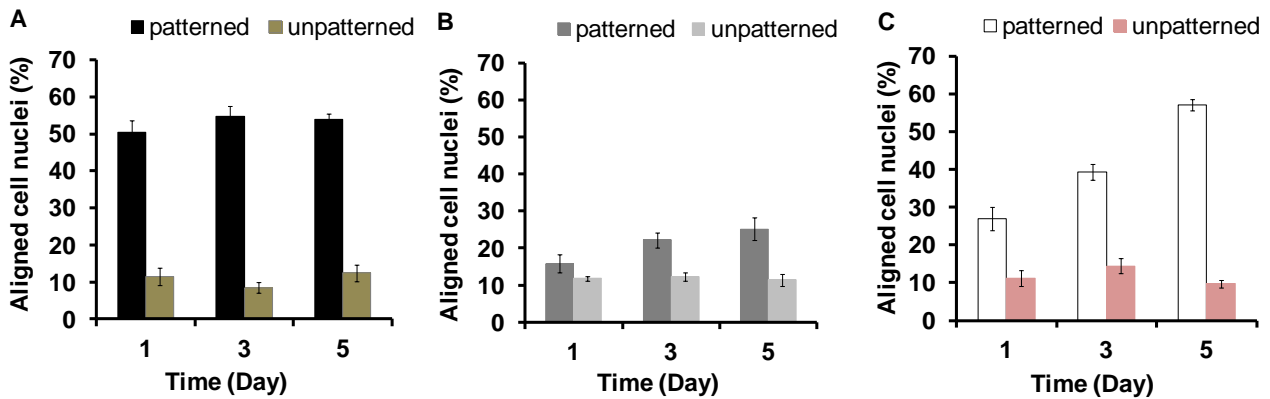


Figure 3: Quantification of cell alignment during the time of culture in patterned and unpatterned zones (A) C2C12 mono-culture, (B) PC12 mono-culture, (C) C2C12 co-culture with PC12.

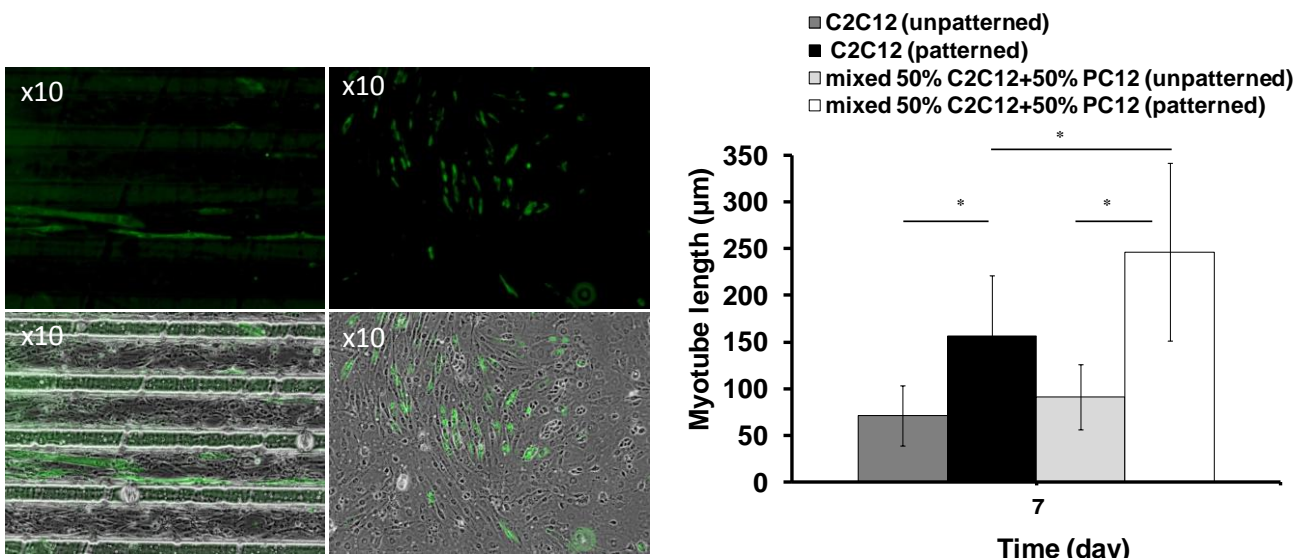


Figure 4: Myotubes formation in co-culture system on patterned (left) and unpatterned (right) regions. Photos in fluorescence (up) and fluorescence merged with visible (bottom).

Figure 5: Quantification of myotubes length when compared between mono-culture and co-culture. Data are expressed for 2 independent experiments and at least 50 myotubes were analyzed (* $p < 0.001$).

observed in the GelMA micropatterned zone compared to the unpatterned region, both in visible and fluorescence observations after double staining of the actin filament (red) with Alexa fluor 546 conjugated phalloidin and the cell nuclei (blue) with DAPI. The quantification of the cell alignment in the different culture systems by the alignment quantification of DAPI stained nuclei as described previously [1] showed significant difference between the patterned and the unpatterned zones (Fig. 3). The cell alignment evolution during the time of culture in the patterned zones was also different in the three culture systems. Thus, while the cells alignment was at 54% and 22% in C2C12 and PC12 mono-cultures, respectively, it increased regularly from 27% at day 1 of culture to 57% at day 5 of culture in the co-culture. In contrast, in the unpatterned zones (Fig. 3) the cells alignment was low at around 11% all along the culture time in the three culture systems. To induce the myoblasts differentiation into myotubes [6], the culture medium was then switched for a differentiation culture medium made of DMEM complemented with 2% horse serum (HS) and 1% antibiotics and the cells were cultured for 7 days. It was observed that the myotubes formation due to myoblasts fusion was significantly improved in micropatterned regions compared to unpatterned regions (Fig. 4). Moreover in co-culture, the presence of PC12 also enhanced significantly the myotubes formation compared to mono-culture (Fig. 5) as shown by the length quantification of the myotubes marked with anti-myosin (MY-32), IgG antibody (primary Ab 1:1000 dilution, Abcam, code ab-7784) and anti-IgG Alexa Fluor 488 conjugated antibody (secondary Ab 1:1000 dilution, Invitrogen, code A-11001).

CONCLUSION

Our work showed that the muscular tissue formation is improved when C2C12 cells are co-cultured with PC12. Therefore, establishing the cellular heterotypic communication with the neural components should be considered as an important way of building a muscular tissue, as it is usually admitted when co-culturing myoblasts with fibroblasts or endothelial cells.

ACKNOWLEDGEMENTS

This work was supported by the World Premier International Research Center Initiative (WPI), MEXT, Japan.

REFERENCES:

1. H. Aubin, J.W. Nichol, C.B. Hutson, H. Bae, A.L. Sieminski, D.M. Cropek, P. Akhyari, A. Khademhosseini, *Directed 3D cell alignment and elongation in microengineered hydrogels*, *Biomaterials*, **31**, 6941-6951, (2010).
2. N.F. Huang, R.J. Lee, S. Li, *Engineering of aligned skeletal muscle by micropatterning*, *American Journal of Translational Research*, **2**, 43-55, (2010).
3. S. Wagner, O.M. Dorchie, H. Stoeckel, J.-M. Warter, P. Poindron, K. Takeda, *Functional maturation of nicotinic acetylcholine receptors as an indicator of murine muscular differentiation in a new nerve-muscle co-culture system*, *Pflügers Archives European Journal of Physiology*, **447**, 14-22, (2003).
4. D.W.J. van der Schaft, A.C.C. van Spreeuwel, H.C. van Assen, F.P.T. Baaijens, *Mechanoregulation of vascularization in aligned tissue-engineered muscle: A role for vascular endothelial growth factor*, *Tissue Engineering Part A*, **17**, 2857-2865, (2011).
5. K. Nagamine, T. Kawashima, T. Ishibashi, H. Kaji, M. Kanzaki, M. Nishizawa, *Micropatterning contractile C2C12 myotubes embedded in a fibrin gel*, *Biotechnology and Bioengineering*, **105**, 1161-1167, (2010).
6. V. Hosseini, S. Ahadian, Ostrovidov S., Camci-Unal G., Chen S., Kaji H., Khademhosseini A., *Engineered contractile skeletal muscle tissue on a microgrooved methacrylated gelatin substrate*, *Tissue Engineering Part A* (2012) (in press).

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