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Growth and differentiation potentials in confluent state of culture of human skeletal muscle myoblasts

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The transitional behaviors of myoblasts toward differentiation were investigated in the cultures at the low and high seeding densities (respectively, $X_0 = 1.0 \times 10^3$ and 2.0×10^5 cells/cm^2). In the culture at the low seeding density, an increase in confluence degree accompanied a decrease in growth potential (R_p), being $R_p = 0.85$ and 0.11 at $t = 48$ and 672 h, respectively. Myoblasts seeded at the high density resulted in the immediate cessation of growth with keeping the low range of $R_p = 0.02$–0.09 throughout the culture. The reduction of R_p led to the generation of three subpopulations of cells in proliferative, quiescent and differentiated states. Close cell contacts in the confluent state of high seeding culture induced cell quiescence to a higher extent with suppressing differentiation.

[**Key words:** Tissue engineering; Myoblasts; Confluent state; Quiescence; Differentiation]

Skeletal muscle myoblasts exhibit a remarkable capacity of self-renewal, which leads to a practical concept of myoblast transplantation for the treatment of degenerative diseases such as muscular dystrophy (1). The myoblast transplantation makes the revolutionary development in recovering damaged myocardial tissue, which lacks self-renewal potential (2). The performance of myoblast transplantation by direct injection of cell suspension into a damaged site of cardiac muscle made significant improvement in its functionality accompanied by the enhancement of left ventricular ejection fraction and muscle contractility (3). However, in the direct injection method, the grafted cells may be easily dispersed, leading to low efficiency of engraftment on host cardiac tissue (4). To overcome this drawback, the employment of prepared myoblast sheet has been proposed as an alternative strategy for grafting (5).

For the preparation of myoblast sheet, the cells are incubated in a confluent state on a thermo-responsive culture dish, and the sheet is recovered as a single layer with intact cell-cell junctions by temperature lowering to 20 °C (6). Such a confluent state of myoblasts allows the close contacts among cells, which is known to initiate the process towards myoblast differentiation (7). The process of myoblast differentiation is associated with the active fusion of mononuclear multiplying cells and the resultant formation of multinuclear myotubes that permanently loose the proliferative ability. Recent studies (8, 9) suggested that the functional efficacy of grafted myoblasts relies on the existing cells with proliferative potential due to their ability to secrete stimulators such as hepatocyte growth factor, vascular endothelial growth factor, pro-angiogenic growth factor and angiogenin. These factors are known to stimulate the angiogenesis in damaged cardiac tissue and enhance the survival of cardiomyocytes. In this context, understanding of populational profiles of myoblasts participating in the proliferation and differentiation in a confluent state is prerequisite for evaluating the quality of prepared cell sheet. In the present study, the cultivations of human skeletal muscle myoblasts were conducted at varied seeding densities, and the transitional cell behaviors were investigated in terms of cell growth potential and gene expressions of cellular state markers.

Human skeletal muscle myoblasts (Lot no. 4F1618; Lonza Walkersville, Inc., Walkersville, MD, USA) were maintained through subculturing in a 75-cm^2 T-flask (Nunclon Delta Flask; Nulgene Nunc International, Rochester, NY, USA) with 15 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA), as described elsewhere (10). The cells experiencing four passages were used as seeds, and the low and high seeding densities were set at $X_0 = 1.0 \times 10^3$ and 2.0×10^5 cells/cm^2, respectively. The cultures were conducted in 8-square well plates (surface area of 10.5 cm^2; Nulgene Nunc International) with a medium depth of 2 mm at 37 °C under a 5% CO_2 atmosphere. The medium change was daily performed.

The confluent degree (C_d), growth potential (R_p) and total cell concentration based on nucleus number (X_T) were estimated with

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respect to the adherent cells during the cultures. The C_d was determined using phase-contrast images, as described elsewhere (11). For determining the R_p and X_T, the proliferative and total nucleus numbers were evaluated by fluorescent staining. The proliferative nuclei were immunostained for Ki67, a nuclear protein that is produced by the proliferating cells in all phases of active cell cycle and down-regulated in differentiated and quiescent states. For staining, the cells on the culture surface were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeabilization with 0.05% Triton X-100 in PBS. After masking non-specific proteins with Block Ace® (Dainippon Sumitomo Pharma, Osaka), the cells were kept overnight at 4 °C with a mouse monoclonal anti-Ki67 antibody (1:250 dilution; Abcam, Cambridge, UK). The cells were then immunolabeled with goat anti-mouse IgG (1:400 dilution; Alexa Fluor 488, Molecular probes, Eugene, OR, USA) accompanied by nuclear staining using DAPI (1:15000 dilution; Molecular probes). Images were captured at randomly selected five positions from each sample using a fluorescence microscope. The Ki67- and DAPI-positive nuclei were counted to calculated the R_p value, which is defined as a ratio of proliferative (Ki67-positive) nucleus number to the X_T value (DAPI-positive nucleus number).

According to the protocol in previous work (10), the cells were subjected to the quantitative real-time PCR to analyze the gene expressions of p130, myogenic factor 5 (Myf5), myogenin and skeletal muscle specific myosin heavy chain 2 (MYH2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The specific primers for the target genes (see supplementary Table S1) were designed using Primer3 software (http://primer3.sourceforge.net/).

To evaluate growth properties after re-seeding, the suspended cells were plated at $X_0 = 1.0 \times 10^3$ cells/cm^2. The number of adherent cells on the culture surface was determined at 24 h of incubation, and the efficiency of cell attachment (α) was obtained as a ratio of adherent cell concentration to the seeding density. The incubation lasted until 48 h to determine the growth potential after re-seeding (R_p') according to the immunostaining procedure as mentioned above.

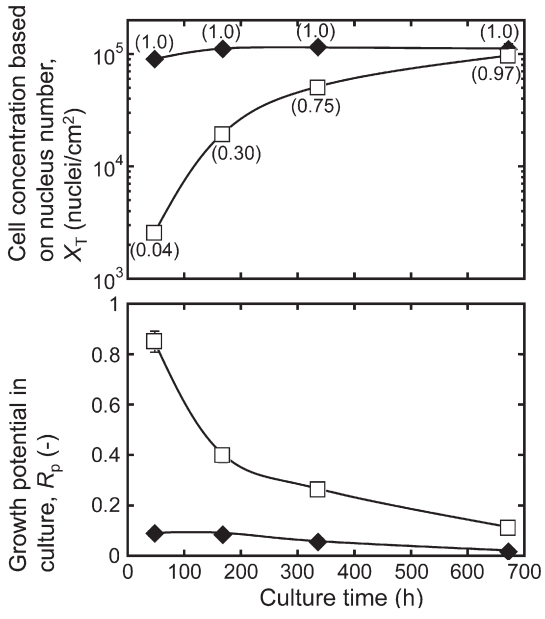

FIG. 1. Time profiles of myoblast concentration and growth potential durin cultures at low and high seeding densities. The open square and close diamond correspond to the cultures at the low and high seeding densities, $X_0 = 1.0 \times 10^3$ and 2.0×10^5 cells/cm^2, respectively. The data were obtained from three independent experiments. The figures in the parentheses represent the values of C_d at the data points.

The cultures of myoblasts were performed in subconfluent and confluent states at $X_0 = 1.0 \times 10^3$ and 2.0×10^5 cells/cm^2, respectively. As shown in Fig. 1, in the low seeding density culture, the X_T increased exponentially with time, accompanying the increment in C_d, and at $t = 672$ h $X_T = 1.0 \times 10^5$ nuclei/cm^2 and $C_d = 0.97$ were achieved. In this culture, the R_p decreased gradually with time. In the high seeding density culture, on the other hand, the X_T at $t = 48$ h was 1.1×10^5 nuclei/cm^2 with C_d close to unity, and no significant variations in X_T and C_d were observed with elapsed time, meaning the cessation of myoblast growth. In addition, the R_p was at a low value of 0.09 even at $t = 48$ h, which was one ninth of that in the culture at the low seeding density, and the low level was kept until the end of culture. These results suggest that cell–cell contacts in the culture play a pivotal role in reducing myoblast growth potential.

To investigate the cellular states in the myoblast cultures, the mRNA expressions of p130, Myf5, myogenin and MYH2 were analyzed. As shown in Fig. 2A, in the low seeding density culture, the expression of p130, an indicator for suppression of cell cycle as well as entering into a quiescence state (12), was enhanced with time, and at $t = 672$ h the level was 1.8 times higher than that at $t = 48$ h. In the culture seeded at the high density, the p130 level was higher throughout the culture compared to that in the low seeding density culture. The expression level of Myf5, which is expressed in actively proliferating myoblasts (13), was almost unchanged until $t = 336$ h, and down-regulated at $t = 672$ h in the culture at low seeding density (Fig. 2B). In the high seeding density culture, the down-regulated expression of Myf5 was observed throughout the culture, giving the negligible expression at the end of culture. The expression of myogenin, which indicates myoblast commitment towards differentiation (13), increased with elapsed time in the culture at the low seeding density, being maximized at $t = 336$ h, which was 36 times that at $t = 48$ h (Fig. 2C). The expression of MYH2, an indicator for phenotypic differentiation of myoblasts (13), was up-regulated at $t = 672$ h in the culture at the low seeding density (Fig. 2D), corresponding to the down-regulation of myogenin. In the high seeding density culture, with culture proceeding, the up-regulation of myogenin was also observed with a maximum at $t = 168$ h, though was still lower than that in the low seeding density culture at $t = 336$ h. The expression of MYH2 showed a slight increase with elapsed time in the high seeding density culture, although the expression level at $t = 672$ h was fairly lower than that in the culture at the low seeding density. The down-regulated expressions of myogenin and MYH2 as well as the up-regulated expression of p130 in the high seeding density culture suggest the suppression of myoblast differentiation with entering into a quiescence state.

To analyze the efficiency of cell attachment (α) and subsequent growth potential of the adhered cells (R_p'), the suspended cells were re-seeded after harvesting from the cultures indicated in Fig. 1. As seen in Fig. 3, for the cells from the low seeding density culture, the α decreased gradually with elapsed time. For the cells from the high seeding density culture, the moderate decrease in α was observed. On the other hand, the R_p' of adhered cells after re-seeding was almost constant around $R_p' = 0.8$ on an average irrespective of the cells from both the cultures. Cruci et al. (14) reported that focal adhesion disappeared along differentiation of C2C12 myoblasts, with loosing filopodia and stress fibers, and this cytoskeletal variations led to loss in the ability of cells to adhere to a substrate. In the present study, the cells attaching on the surface after re-seeding were considered to preserve the proliferative ability because of excluding the non-adhered cells arising from differentiation. In this context, in the culture at the low seeding density, the decrease in α, in accordance with decreasing R_p as seen in Fig. 1, indicated that the non-proliferative cells progressed towards the process of myoblast differentiation which was confirmed by the increments in myogenin and MYH-2 expressions with elapsed time (Figs. 2C and D). To contrast,