Fine Adhesion Patterning and Aligned Nuclei Orientation of Mesenchymal Stem Cell on Narrow Line-Width of Silicone Rubber Implanted by Carbon Negative Ions*

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Fine cell-adhesion patterning and nuclei orientation control on silicone rubber were investigated by limiting line-width of carbon negative-ion implantation. The ions were implanted at 3×10^{15} ions/cm² and 10 keV through masks of ridge-pattern and rectangle-pattern with various slit apertures of 0–40 μ m in width. After 2 days culture, nuclei of rat mesenchymal stem cells were stained with fluorescent dye to evaluate the nuclei positions at each tip ridge-region and the relative angles between the major axis of ellipse-shape nuclei and the implanted line-region. Results showed the individual nucleus arrangement along the narrow line-widths of 3–12 μ m and the gathered one on the wider line-width. Number of nuclei orientation in the angle range of 0°–10° increased to 79% on the line-width of 10 μ m. As a result, the fine cell-adhesion patterning and nuclei orientation could be achieved by decreasing the implanted line-width to 10 μ m.

1. Introduction

Improvement of cell adhesions on polymeric materials have been purposed by using ion implantation with both ion-types of positive and negative^{1–5}. Negative-ion implantation techniques have been used to modify polymer surface for improvement of cell-affinity because of its advantage of “charge-up free”^{6–8}. For cell culture, carbon element is selected because of its harmless to cell and matching to polymer-lattice. As previous work, we modified the hydrophobic polymer of silicone rubber (SR) by using carbon negative-ion implantation^{3–5}. XPS analysis showed the formations of oxygen functional groups such as carbonyl and hydroxyl on the ion-induced defects after the implantation⁴. Such formed groups having negative-polar characteristic refer to the hydrophilic properties, leading to the lowering of water contact angle of the implanted SR from about 100° to 86°^{3,4}. These effects promoted the adsorption of extracellular matrix (ECM), necessary adhesive proteins for cell adhesion, on the implanted area^{3,4}. By using micro-pattern mask with slit-aperture width of 50 μ m, we could pattern the adhesions of nerve cells and mesenchymal stem cells (MSC) on the implanted regions^{3,5}. Moreover, we had investigated the trial differentiation of MSC into neuron with retaining adhesion pattern⁵. However, we obtained only the short neurite outgrowth due to random cell-orientation and the gathered cells on this implanted line-width. For the artificial nerve network, the cell-adhesion patterning with cell orientation control for long neurite length is required.

In this work, the fine adhesion patterning and nuclei orientation arrangement of MSC on the C⁻-modified SR

were investigated by decreasing the implanted line-width.

2. Material and Methods

Silicone rubber sheets (SR, Fuji systems, Japan) with thickness of 0.5 mm were implanted by carbon negative ions. Our negative-ion implanter is composed of the neutral and ionized alkaline metal bombardment type heavy negative-ion source (NIABNIS)^{9,10}, the electro-magnetic mass separator and the implantation chamber with having beam limiter of 11.28 mm-diameter. The carbon negative-ion beam was operated at dose of 3×10^{15} ions/cm², energy of 10 keV with a lower current density than 400 nA/cm² to prevent surface melting under residual gas of about 2×10^{-4} Pa. For fine adhesion patterning and nuclei orientation control, the specific masks with an area size of 5 $\times$ 10 mm² were used as shown in **Fig. 1**, where (a) ridge-pattern was consisted of slit apertures in the width range of 0–40 μ m with a tip angle of about 1.1° and (b) rectangle-pattern was consisted of various slit apertures of 5, 10, 20, 30 and 40 μ m in width. After sterilization, rat mesenchymal stem cells (MSC, R492-05, Cell Applications Inc., USA) with amount of 1.7×10^3 cells were cultured on each sample in Dulbecco modified Eagle medium (DMEM, Nissui, Japan) containing 5% horse serum (HS, Gibco, New Zealand), 5% fetal bovine serum (FBS, Trace Scientific Ltd., Australia) and sodium hydrogen carbonate (1.8 mg/ml, Wako, Japan) with antibiotics of penicillin G and streptomycin for 2 days under 5% CO₂ at 37°C in an incubator. Cell-nuclei positions and their orientations were observed by fluorescent microscopy with staining of 4', 6-diamino-2-phenylindole (DAPI, Sigma-Aldrich, Japan). **Fig. 2** shows schematic diagrams for evaluation of (a) the top cell or the individual nucleus at tip region on each ridge line and (b) the relative angle between the major

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Fig. 1 Schematic diagrams of special micro-pattern masks of: (a) ridge-pattern and (b) rectangle-pattern.

Fig. 2 Schematic diagrams for evaluation of: (a) the top cell or the individual nucleus at tip region on each ridge line; and (b) the nuclei orientation on each rectangular line.

Fig. 3 MSC arrangement on the tip region of ridge-pattern of C^-/SR at 3×10^{15} ions/cm² and 10 keV after 2 days culture with 4 repeat-samples: (a) fluorescent image; and (b) numbers of nucleus arrangement on each tip line-width.

axis of the ellipse-shape nuclei and the implanted line-width. The line-width for controlling fine adhesion-pattern of MSC was evaluated from the position and orientation of the individual nuclei arrangement on each line-width.

3. Results and Discussions

MSC arrangement on the tip region of ridge-pattern of C^- -implanted SR (C^-/SR) at 3×10^{15} ions/cm² and 10 keV after 2 days culture is shown in Fig. 3, where (a) is fluorescent image and (b) is numbers of nucleus arrangement on each tip line-width. Adhesion arrangements of the individual cells and the gathered cells depended on the implanted line-width. In Fig. 3 (a), the bright dots are corresponded to cell nuclei. The arrangement of one nucleus from the individual cell elongated along the implanted line-width in the range of 2–5 μm was found. Generally, the size of spread MSC is in the range of 50–100 μm. The adhesion of cells on the narrower line-width than cell size was considered to be due to two effects. One is the improved adsorption of ECM on the implanted regions and other is the small amount of ECM

adsorbed on the interface area between the implanted and unimplanted regions^{3,4}. The elongated adhesion along the implanted region and a little spread adhesion around the boundary regions resulted in the observed cell adhesion on the narrower line-width than the cell size.

In this work, we could divide the arrangements of nuclei into two types of individual and gathering as shown in Fig. 3 (b). Nuclei at tip-region were totally 79 ± 6 nuclei for 83 implanted lines. The individual arrangement was only 25 ± 2 nuclei. The nuclei number increased with an increase in the line-width from 3 to 12 μm. The average line-width for individual arrangement was about 8 μm. On the wider line-width of 12 μm, the arrangement of group nuclei from the gathered cells was found. The observed nuclei had an elliptic shape with size of about $10 \times 20 \mu\text{m}^2$ in axes of minor and major, respectively. Direction of the major axis of the cell nuclei was parallel to the implanted line. Then, this is corresponding to the most population of individual cell arrangement with the same direction on the line-width of about 8 μm.

Fig. 4 Nuclei orientation on the various line-widths of rectangle-pattern of C^-/SR with 3 repeat-samples: (a) fluorescent image of MSC on line-widths of 5–30 μm ; and (b) histogram of nuclei orientation on each line-width.

Nuclei orientation on the various line-widths of rectangle-pattern of C^-/SR is shown in **Fig. 4**, where (a) is fluorescent image of MSC on line-widths of 5–30 μm and (b) is histogram of nuclei orientation on each line-width. Number of adhered cells increased with an increase in the line-width as shown in **Fig. 4** (a). Similar to the results of ridge-pattern, the individual cells were found on the narrow line-widths of 5–10 μm . Number of nuclei, especially individual type, rapidly increased from 40 to 250 nuclei/ mm^2 with an increase in line-width from 5 to 10 μm , and that of all nuclei saturated at 320 nuclei/ mm^2 for the wider line-width than 20 μm (data not shown). From **Fig. 4** (b), percentage of the observed nuclei depended on the relationship between the line-width and the nuclei orientation angle. The highest percentage of nuclei orientation for all line-widths was at the angle less than 10° (0° – 10°) due to the elongated cell along the line-region, resulting in the self-orientation of the nuclei in the same direction. As previous work for culturing MSC on the line-width of 50 μm^5 , the percentage at this angle was only 28%. With the decrease in line-width from 40 to 10 μm , the percentage of orientation at this angle increased from 45% to nearly 80%. Regarding to the large size of MSC, the outgrowth of neurite from MSC differentiation should be longer than 100 μm . However, the previous observed neurite on the 50– μm line-width of C^-/SR was only in the length range of 50–80 μm due to the gathering of cells on each line-width⁵. At the angle less than 10° and the narrow line-width of 10 μm , the cell elongation also increased the cell-to-cell distance, resulting in the possibility to improve the outgrowth of long neurite.

4. Conclusions

The fine adhesion patterning and nuclei orientation control of MSC on SR was investigated by decreasing the line-width of carbon negative-ion implantation. One nucleus from the individual cell arranged and elongated

along the narrow line-widths of 3–12 μm while the group one from the gathered cells were on the wider line-width than 12 μm . The observed orientation of cell nuclei was almost in the same direction of the narrower line-width than 40 μm . For the relative angle less than 10° to the line-region, percentage of nuclei orientation could be increased to nearly 80% on the implanted line-width of 10 μm . As a result, we achieved the fine adhesion pattern and nuclei orientation control of MSC on SR by decreasing the implanted line-width to 10 μm .

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