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Surface Topographic Effect on Mesenchymal Stem Cells in Tissue Engineering

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In the field of tissue engineering, researches have been actively conducted to regulate stem cell fate by understanding the interaction between cell and materials. This approach is expected as a promising therapeutic method in the future medicine by utilizing differentiation of stem cells into desired cells or tissues using biomaterial. For this regenerative medicine, there exist lots of attempts to construct optimized structures of various shapes and sizes that can regulate the stem cell fate. In this review, we will empathize the topographic effect as stem cell niche on the mesenchymal stem cell (MSC) response (cell attachment, proliferation, and differentiation) according to the shape and size of the structure of the substrates, and comprehensively analyze the importance and the effect of shape and size of the surface topography.

Key Words Tissue engineering · Regenerative medicine · Mesenchymal stem cells · Topographic effect.

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Introduction

The combination of stem cells and biomaterials has provided potential for regenerative medicine. The key point of the approach is to differentiate stem cells into specific cell lineages or tissues using biomaterials (1). This tissue-engineered approach requires an understanding of the characteristics about both of the stem cells and the biomaterials and the interaction between them. In the case of tissue-engineered applications and conventional in vitro cell experiments, a 2D culture system provides advantages of easily supplementing oxygen and nutrients (2). Investigating the cell responses on the surface of the implant using the 2D culture system, studies on the interaction of stem cells and surface topography have been conducted.

Mesenchymal stem cells (MSCs) have been focused because they can be easily harvested from the human body and have capability of differentiating into various cells or tissues for surgical procedures. Many efforts to investigate and simulate stem cell niche exist, as it is known that cell responses are affected by the surrounding microenvironment and sensing at the same time (3). An external local microenvironment of stem cell, called stem cell niche, affects stem cell behavior such as adhesion, proliferation, and differentiation. The above-mentioned surface topography corresponds to the physical component among the three components (chemical, physical, and cellular components) that make up the stem cell niche (4). Therefore, observing the

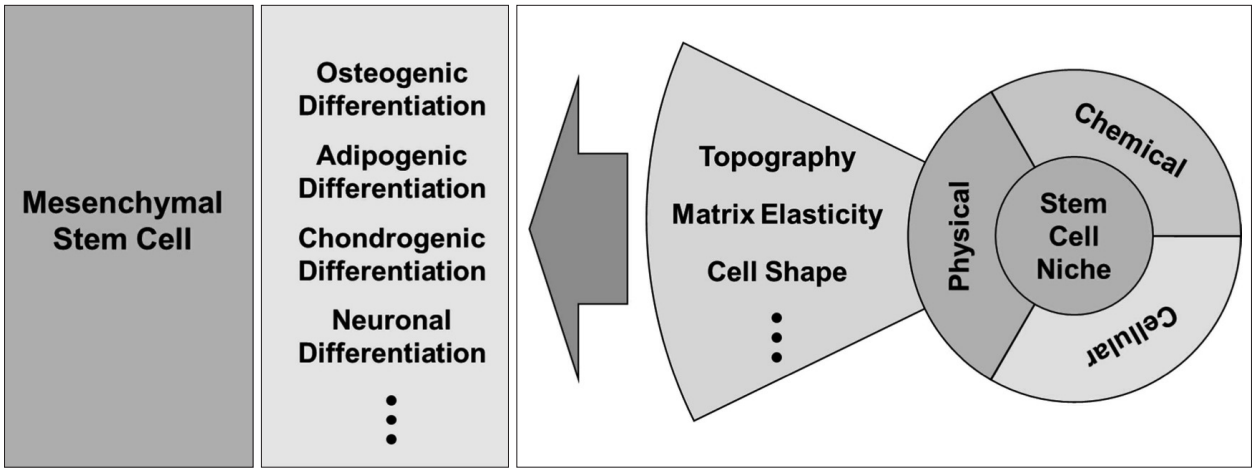

Fig. 1. Multipotent of mesenchymal stem cell and physical component of stem cell niche in tissue engineering.

response of stem cells on various surface topography is crucial for understanding their interactions, thus developing tissue-engineered applications (Fig. 1).

There have been many attempts to regulate stem cell fate based on the understanding the interaction of stem cells and the microenvironment (5). Previous researches demonstrate that stem cell fate is mostly influenced by biochemical components such as growth factor, protein, and hormones and also by physical components such as topography and stiffness (1, 6-8). Furthermore, it has been shown that topographic effect alone without biochemical factors can also affect stem cell fate (9, 10). As nanotechnology develops further, it has become possible to create nanostructures that are much smaller and more precise than ever before. Electron beam lithography is a typical method and the nanostructures down to below 10 nm can be patterned on the surface by this method (11, 12). Additional nanofabrication techniques include top-down and bottom-up processes to fabricate nanoscale and microscale structures. By using combinations of different fabrication techniques, it is possible to produce various nanostructures of desired shape and specific size. Those techniques form a basis for defining topography that effectively controls the cell responses.

We review the researches about the response of MSCs to surface topography and the related fabrication processes with respect to tissue engineering. In order to understand the mechanism of regulating stem cell fate induced by topographical effect, size and shape of topography is emphasized. Then, we consider the remaining challenges in using MSCs for regenerative medicine.

Mesenchymal Stem Cells (MSCs) in Tissue Engineering

Stem cells refer to the cells that are capable of differentiating into various cell lineages and proliferating while keeping undifferentiated state at the same time. The characteristics of stem cells can be clearly observed in embryonic stem cells. The pluripotency and self-renewal ability of embryonic stem cells can be used in combination with tissue engineering to show high potential for regenerative medicine (13). However, embryonic stem cells also have ethical issues as well as potential problems of differentiating into cancer cells (14).

MSCs are attracting attention as one of the alternatives to embryonic stem cells. The MSC was first found from bone marrow in the early 1960s (15). Since MSCs are capable of differentiating into muscle tissue, fat, cartilage, and bone tissue, and can be easily harvested from the patient's body, studies on their tissue engineered application have been actively carried out (16, 17). There are three requirements for the use of stem cells in tissue engineering (18). A large number of cells should be collected and tissue engineering products from them should be safe when they are used on the human body. They should also maintain undifferentiated state until it is used for tissue engineering. It should be noted that some challenges remain as the amount that can be collected from the human body is limited, and the state of the MSCs is affected by the age and physical condition of the patient (19, 20). To meet those requirements, researchers have attempted to regulate stem cell fate based on the understanding of the functions of stem cell niche in the tissue engineering.

Fabrication Techniques for Surface Topography

With the development of nanotechnology, it has become possible to fabricate various structures that are comparable in the size with extracellular matrix (ECM). The most commonly

used structures in related studies are ridge/groove, pit, pillar, and so on. In order to form the topography that simulates the stem cell niche, the patterning methods such as lithography, etching, pattern transfer, and direct milling are used either by themselves or in their combinations depending on the substrate material or the desired size and shape of the structures.

The fabrication techniques for patterning can be categorized in general into top-down and bottom-up methods (Fig. 2). Top-down fabrication is a subtractive process where part of the material is patterned to make structures with desired shape and size. Photolithography and direct milling are the representative methods for top-down process. On the other hand, bottom-up fabrication is an additive process where atoms and molecules are assembled to make structures. Self-assembled nanostructures, bottom-up synthesis such as nanowires and nanodots are the representative methods for bottom-up process.

The top-down fabrication benefits from the precise control of energetic beam such as photon, electron, and ion to put the desired patterns in exact location. While it is widely used in microelectronics manufacturing, its resolution is limited by the very nature of beam control process such as a mask used for a photon beam that does not have electric charge and an electromagnetic lens used for focusing electron and ion beams with negative and positive charges, respectively. Smaller structures than a few hundred nanometers or below would need a highly controlled and costly top-down process for large scale fabrication or it may make more sense to take advantage of bottom-up fabrication with chemically controlled self-assembled process in a potentially atomic and molecular scale precision. The disadvantages of the bottom-up self-assembly process is the ability to pattern the structures of arbitrary shape as the process is limited by what nature allows us to produce and the limitation of large scale uniformity for actual manufacturing. These limitations from the top-down and bottom-up processes pushed a development of hybrid nanofabrication techniques such as nanoimprinting and soft lithography where nanoscale structures are fabricated by slow and costly top-down process such as electron beam lithography (EBL) or low cost bottom-up fabrication process such as colloidal self-assembly and then replicated by transferring the nanopatterns with soft lithography process.

Among the top-down processes, the direct milling can be used to cut the surface of a substrate in a desired structure. Typical methods include focused ion beam and laser milling. Photolithography is a core technique of semiconductor production

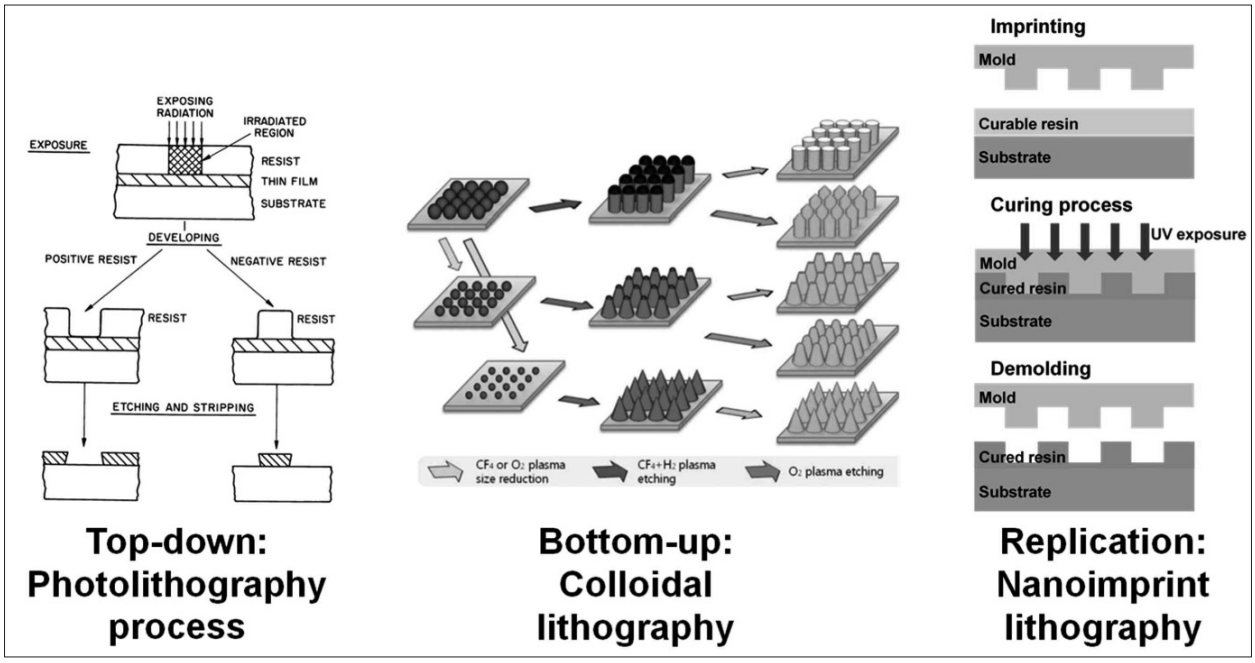

Fig. 2. Representative schematic of fabrication techniques **(Top-down: Photolithography process)** The schematic diagram shows the process of photolithography including exposure through mask after resin coating, development, and post process of etching and stripping. The residual resin pattern after development is used to fabricate surface topography as mask (21). *Reprinted with permission from Ref. 21. Copyright 1983, American Chemical Society.* **(Bottom-up: Colloidal lithography and post processes for the fabrication of nanopillars)** At the left side, nanoparticles are aligned as hexagonal closed packed monolayer by self-assembly. From the left, the underlying substrate is modified to pillar array using the monolayer as an etch mask. Size reduction of nanoparticle with post processes to control the size of mask makes the altered shape of nanopillars (23). *Reproduced from Ref. 23 with permission of The Royal Society of Chemistry*. **(Replication: Pattern transfer process using UV-Nanoimprint Lithography)** The schematic diagram shows the process of ultraviolet (UV) nanoimprint lithography (NIL) including resist coating, imprinting mold into the resin, UV curing and demolding process.

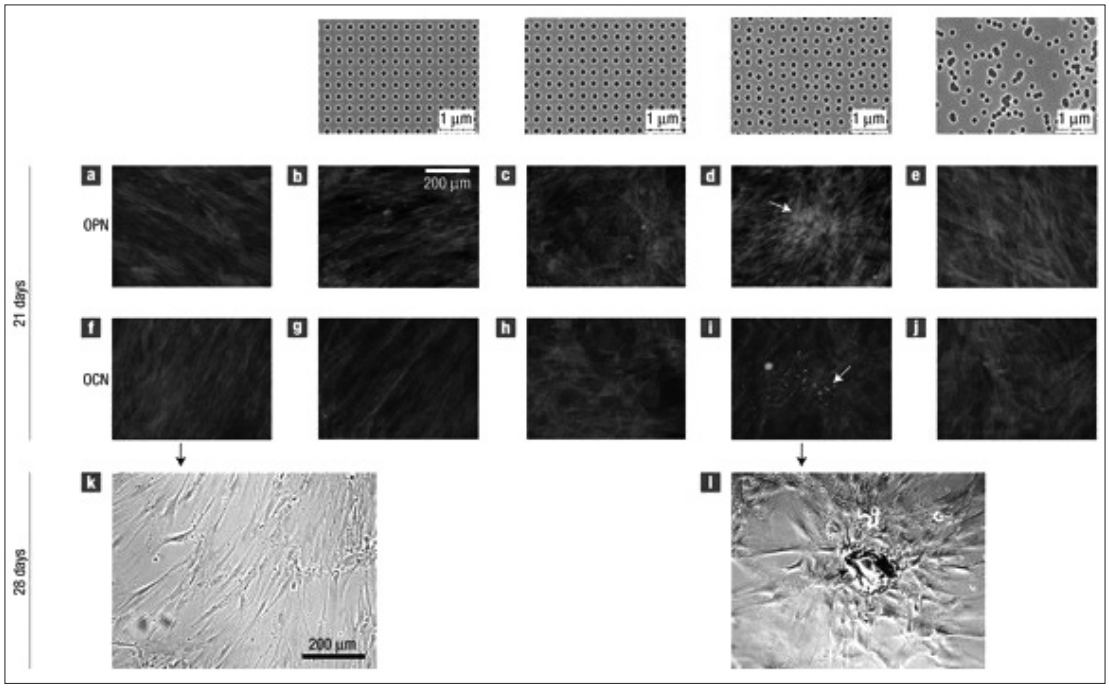

Fig. 3. Representative result of topographic effect. SEM images of nanopit arrays from ordered to disordered arrangement (upper side), fluorescence microscopy images of actin (red) and osteopontin (OPN)/osteocalcin (OCN) (green) as bone-specific ECM protein (bottom side): nanoscale disordered pit array induced MSCs to produce bone mineral without biochemical environment (9) *Reprinted by permission from Macmillan Publishers Ltd: Nature Materials (ref. 9), copyright 2007*.

method. The process of photolithography includes photoresist coating on Si wafer, UV exposure, development, and post process (21). The photoresist made of a polymer resin is spin-coated onto the Si wafer, then exposed through the mask pattern to react under UV light. The resin dissolves selectively in a developer because the solubility of resin changes by UV exposure. The development of semiconductor fabrication technology has led to the optimized process so any types of structure can be generated in microscale with relative ease.

However, when going down to the nanoscale, there is a resolution limit due to the diffraction of light depending on the wavelength of the UV used as the light source. To overcome the resolution limit of photolithography, the methods of using shorter wavelength sources such as electron beam and extreme ultra violet (EUV) have been investigated. Electron beam lithography (EBL), which is used in the prototyping for many nanoscale experiments, can form a nanoscale pattern down to 10 nm or less on a substrate (11, 22). In contrast to conventional photolithography where exposure through a mask defines a pattern in the process, EBL enables a direct patterning without using a mask by utilizing a computer aided design (CAD) tool to design a pattern and then by using a X, Y axis controller or an electron beam blanker attached to scanning electron microscope to provide positioning capability of the electron beam. Patterns made using these lithography processes can be used directly or in combination with the other methods of post processing such as deposition or etching.

These post processes can be used to introduce additional topography on the patterns fabricated with the methods described previously. The deposition methods include chemical vapor deposition (CVD) and physical vapor deposition (PVD). The CVD method is a technique of forming a thin film by reacting at the surface of a substrate in ambient source gas using external energy such as heat, plasma, and light. PVD uses vaporizing or ionizing a metal or a dielectric material in a vacuum to form a thin film on the surface of a substrate. Depending on how materials are transferred from target material to a substrate, the techniques are called an evaporation coating if it involves vaporization of source material, a sputtering if it utilize ion bombardment to sputter species off from the source, or an ion plating if glow discharge plasma is initiated to enhance the energy of the species for better deposition. Etching is categorized into wet etch and dry etch. Wet etch is immersed in a reactive solution as the name implies, and dry etch is a method of selectively removing the area that is not shielded with a mask by using a plasma process.

Self-assembly is a fundamental method for the bottom-up process and has the advantage of high resolution by directly using molecules or nanoparticles. Colloidal lithography forms a pattern through the self-assembly of colloidal polystyrene

Table 1. Topographical effect and fabrication methods

Topography	Feature	Fabrication	MSC behavior	Ref.
Roughened surface	Micropatterned nanorough area	Combined ion beam/ chemical etching process	hMSCs from bone marrow has an affinity with planar surface than nanorough surface	(33)
Ridge/groove	(Micro) 2−15 μm (Nano) 650 nm pitch	Lithography and dry etching, Multi beam intereference milling	15 μm and 2 μm ridges enhance adipogenic and osteogenic differentiation each. 650 nm pitch enhances both differetiation	(28)
	350 nm width, 700 nm pitch	Nanoimprint	Elongation and alignment on nanostructures. Up-regulation of neuronal markers	(34)
Pit	120 nm diameter, disordered	Electron beam lithography	Osteogenic differentiation without inducing media	(9)
Pillar	500 nm diameter 3, 5, 10 μm pitch	Electron beam lithography and pattern transfer	Larger pillar-to-pillar distance enhances morphological changes and neuronal differentiation	(10)
	(Micro) ~2 μm diameter, (nano) ~20 nm tip	Photolithography, Wet etching	Cell aggregation on nanopillar enhanced osteogenisis.	(35)
Nanotube	15, 20, 30, 50, 70, 100 nm diameter	Anodization	Smaller nanotubes enhance MSC responses of adhesion, proliferation, and osteogenic differentiation	(30)
	30, 50, 70, 100 nm diameter	Anodization	Small nanotube promotes cell adhesion and large nanotube enhances elongation and osteogenic differetation	(29)

nanospheres and then uses the bottom-up monolayer arrangement as a mask to define the nanoscale topography. The monolayer can be in the form of a hexagonal close packed (HCP) structure. Using this monolayer as a mask, a structure of nanopillar or nanopit array can be generated through the post process of etching (23).

In addition to the exposure or self-assembly based methods, nanoimprint lithography (NIL) is relatively new and provides a hybrid approach of taking advantages from both methods to form nanopatterns (24). Nanoscale patterns meticulously prepared by electron beam lithography or self-assembly based process can be replicated by transferring the patterns by stamping onto the UV curable resin coated on the surface of silicon wafers. Soft lithography leverages similar principle and surface chemistry to control the transfer of the nanostructures from a mold onto polymeric resin materials (25). Capillary force lithography is one of the representative methods. (26, 27). A few drops of UV resin are applied to the surface of the nanostructure. The resin fills the gap of the nanostructure by a capillary force and then it is cured and separated to produce replicated structure. Materials such as Polydimethylsiloxane (PDMS) can be used and cured by heat and then separated to obtain a replica of nanostructures.

Surface Topographic Effect

Shape and size of structures on the substrate have been widely known as factors of topographic effect (6, 9, 28-30). Natural ECM structures are composed of complex topography of entangled fiber and porous structure in nanoscale (31) and the commonly used forms such as a roughened surface, pillar structures, and ridge/groove are inspired from the natural structures. These types of topography from various fabrication techniques affect the regulation of stem cell fates (Table 1). The first observed behavior of MSCs is morphological change. MSCs are elongated or spread on the surface along with the shape and size of underlying topography. The morphological changes generally accompany cytoskeletal reorganization and stress. The cytoskeleton tension and reorganization with focal adhesion are the key factors of topographic effect investigated so far (32). We classify the previous studies according to the type of surface structure and classify the effect of target cell reaction and structure type. In the case of patterning, anisotropic and isotropic structures are considered to reflect the response of MSCs. Anisotropic pattern has ridge/groove structures, and isotropic pattern has structures such as pit, pillar, and nanotube.

Roughened surface

In recent experiments using micropatterning to observe the reaction of human mesenchymal stem cells (hMSCs) from bone marrow on a substrate with a planar surface and a roughened surface, MSCs has shown better adhesion on planar surfaces than roughened surfaces (33). This research described inhibition of focal adhesion on nanoroughened area and suggested the random walk model for analysis of organization of cells on the micropatterned surface.

Roughened surfaces have been used since the early stages of the study on surface morphology and cell response as the roughened surface is relatively easier to fabricate. Roughness on the surface is isotropic and randomly disordered with its characteristic typically defined as the mean value of the height along the z-axis. The cell response on the roughened surface focuses on the area where the surface is defined and measured. Randomly roughened surfaces have different sharpness and smoothness of the protrusions on the surface, and height and spacing of the protrusions, which makes clear conclusions rather difficult as the roughness values (Ra) are averaged over the surface.

Ridge/groove

The effect of ridge/groove structure on cell activity is mainly classified according to the distance between the ridges and the size of the ridges. Such a structure can be made either directly by beam based machining methods such as ion or laser micromachining or indirectly by transferring a pattern of a mold formed by lithography and etching onto a biocompatible polymer. Abagnale et al. analyzed osteogenic and adipogenic differentiation of MSCs isolated from lipoaspirate using various sizes of ridge/groove structures (28). The research has shown that a specific size in microscale promoted the differentiation of stem cells. In microscale, osteogenic differentiation was enhanced on 2 μm ridge and adipogenic differentiation was enhanced on 15 μm ridge. In nanoscale, both osteogenic and adipogenic differentiations were amplified on the ridge/groove structures of 650 nm pitch.

Since ridge/groove is an anisotropic structure, it has a directional effect on cell attachment and maturation. It has also been used for experiments requiring specific orientation and shape of cell arrays such as differentiation of muscle cells and nerve cells. Yim et al. induced hMSCs from bone marrow to differentiate into neuronal-like cells on the nanograting structure with 350 nm width (34). They demonstrated neuronal differentiation of MSCs on the nanograting associated with morphological changes and emphasized the significance of nanotopography.

Pit array

According to Dalby et al.'s results (Fig. 3), not only the size but also the arrangement of nanostructures are important for osteogenic differentiation of stem cells (9). The surface morphology of the substrate used in the experiment was changed by varying the diameter and depth of the nanopit on the surface so that the resulting osteogenesis was compared. This study is considered as a representative example showing the importance of topographic effects as it demonstrated that osteogenesis can be induced in the specific patterns of square array and disorder levels even in the absence of specific growth factors.

Pillar array

Brammer et al. demonstrated the role of nanopillar that affects osteogenic differentiation of MSCs from rat bone marrow (35). They found that nanopillar increased cell attachment, growth and cell aggregation. Furthermore, the cell aggregation induced on the nanopillar enhances osteogenic mineralization. Recently, researchers have reported that controlling the distance of the pillar array can regulate the spread of the filopodia to promote neuronal differentiation of adipose-derived stem cells (10). In order to fabricate the PDMS nanopillar array used in this experiment, a nanopit array mold was fabricated using EBL and etching process, and a pattern was transferred through soft lithography to fabricate the nanopillar structure. In this case, the nanopillar was utilized to induce cell elongation and this morphological change was shown to regulate the stem cell fate.

Nanotube

Tubular arrays have been extensively studied as a means to make porous oxide film on the surface of a Ti alloy, a material frequently used as an implant. For the in vitro studies, titanium oxide nanotube is formed through the anodization process or etching with a porous anodized aluminum oxide (AAO) as a template. In experiments using TiO2 nanotubes, the response of MSCs from bone marrow to various sizes was shown to be different (29, 30). Park et al. observed that adhesion, proliferation, and osteogenic differentiation of MSCs were enhanced in nanotubes of 15 nm diameter (30). However, the results of Oh et al. using nanotubes indicated that osteogenic differentiation was enhanced for larger diameters (29). The two experimental results were not exactly consistent and it would be hard to keep the detailed environment of each experiment same, but these results suggest the necessity of systematic approaches in the development and design for tissue engineered application.

Summary and Conclusion

The development of bio-alternative materials for regenerative medicine through the combination of stem cells and biomaterials lays the foundation for future medical technology development. This requires a deeper understanding of the interaction between stem cells and biomaterials. Activities such as differentiation and attachment of MSCs are influenced by the local microenvironment called stem cell niche. The fabrication of nanostructures opens up the possibility for systematic approaches in mimicking extracellular environments. It has

been found that by producing a specific surface topography, the differentiation of MSCs can be induced or directed with or even without the biochemical influence. MSCs are differentiated into osteoblast and adipocyte as they spread. In addition, neuronal differentiation has been observed to occur through elongation and alignment. Though it is known that the formation and distribution of intracellular skeleton are affected by focal adhesion and tension, thus determining cell morphology and stem cell fate, the regulatory mechanism is still not clearly understood. However, as can be seen from many earlier results, the topographic effect appears to modulate the stem cell fate by affecting the signal pathway through focal adhesion.

For regenerative medicine, it is necessary to systematically approach the effects of stem cell niche, especially for the physical components that can be controlled by various fabrication technologies. Many experiments have been conducted in this regard, but there are many cases in which the experimental results are not sufficient to draw conclusive explanation on the topographic effect. In order to understand the regulatory mechanisms of stem cells by topographic effect, we need to analyze the features of each nanostructure as well as the function of stem cells, and apply the systematic approach to control the experimental environment clearly.

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