TiO_2 Nanotube Structures for Enhanced Cell and Biological Functionality

Karla S. Brammer, Seunghan Oh, Christine J. Frandsen, and Sungho Jin

Nanostructures have pronounced effects on biological processes such as growth of cells and their functionality. Advances in biomaterial surface structure and design have resulted in improved tissue engineering. Nanotechnology can be utilized for optimization of titanium implants with a formation of vertically aligned TiO_2 nanotube arrays on the implant surface. The anodic oxidation of the titanium implant surface to form a TiO_2 nanotube array involves electrochemical processes and self assembly. In this paper, the mechanism of nanotube formation, nanotube bio-characteristics, and their emerging role in soft and hard tissue engineering as well as in regenerative medicine will be reviewed, and the beneficial effects of surface nanotubes on cell adhesion, proliferation, and functionality will be discussed in relation to potential orthopedics applications.

INTRODUCTION

Nanostructured biomaterials have recently received much attention due to their large surface area and the higher degree of biological plasticity compared to microscale or macroscale surface structures. In terms of biomaterial development on implants such as Ti, the cellular response can be affected by topographical circumstances. There is a growing body of data that shows how cells respond positively to nanotopography.[1–13] It has been proven that cells sense and react to nanotopography, in vitro as well as in vivo by exhibiting changes in cell morphology and proliferation, cytoskeletal organization, signaling and gene expression.[1–13] The study of cell interactions with nanotopography is a rapidly expanding field. By fabricating nano-features upon biomaterial surfaces, one provides interacting features that are on the same scale as cell features. Looking at what happens on different nanotextures can help to uncover signaling pathways that promote the desired cellular response for advanced tissue engineering therapies.

Titanium and its alloys have been widely used as implantable biomaterials because of their superior mechanical properties, a native oxide surface layer that provides corrosion resistance, and biocompatibility. In our recent studies of nanomaterials and nanoscale surface structures, vertically aligned TiO_2 nanotubes have become one of the primary candidates that can provide a direct control of many types of cell and tissue behavior.

How would you…

…describe the overall significance of this paper?

Titanium implants have been optimized with a TiO_2 nanotube surface modification which has beneficial effects on cell adhesion, proliferation, and functionality for advanced tissue engineering and orthopedic applications.

…describe this work to a materials science and engineering professional with no experience in your technical specialty?

Advances in biomaterial surface structure and design have resulted in improved tissue engineering. In particular, we have utilized nanotechnology for developing vertically aligned TiO_2 nanotube arrays on titanium implant surfaces. These nanostructures have pronounced effects on biological responses such as cell growth and functionality.

…describe this work to a layperson?

In terms of biomaterial development for implants such as titanium, the biological response can be affected by topographical circumstances. As we change the surface features we can change how the body, cells, and other parts of biology respond for the desired reaction to the implant for improving implantation.

FORMATION MECHANISM OF TiO_2 NANOTUBES ON TITANIUM

There are several methods of producing TiO_2 nanotubes by anodization. The electrolytes are typically fluorine-ion containing electrolytes such as a dilute 0.5% HF solution. The formation of TiO_2 nanotubes in fluorine-ion based electrolytes generally occurs as a result of three simultaneous processes: the field assisted oxidation of Ti metal to form TiO_2, the field-assisted dissolution of Ti metal ions in the electrolyte, and the chemical dissolution of Ti and TiO_2 due to etching by fluoride ions, which is enhanced by the presence of H^+ ions.[14] TiO_2 nanotubes are not formed on the pure metallic Ti surface but on the thin TiO_2 oxide layer naturally present on the Ti surface. Therefore, the mechanism of TiO_2 nanotube formation is related to oxidation and dissolution kinetics.

As is well known, metallic Ti naturally contains a stable, passivation surface layer of TiO_2 that tends to inhibit further chemical reactions to occur on the Ti surface. When the passivation layer is damaged the layer is generally restored quickly. This restoration reaction occurs when the TiO_2 layer is in contact with air or water. This reaction produces TiO as well as hydrogen gas, according to the following reaction:[15]

$$Ti_{(s)} + 2H_2O_{(g)} \rightarrow TiO_{2(s)} + 2H_{2(g)} \quad (1)$$

When the Ti surface is in contact with the aqueous fluorine containing

electrolyte solution, the TiO_2 layer forms rapidly. The detailed dissolution mechanism of TiO_2 with regard to the formation of TiO_2 nanotubes in fluorine-containing solutions has been proposed to be sufficiently described by the following reaction:[16]

$$TiO_{2(s)} + 6F^-_{(soln)} + 4H^+_{(soln)} \rightarrow [TiF_6]^{2-}_{(soln)} + 2H_2O_{(soln)} \quad (2)$$

In this process, an intermediate layer (TiF_6^{2-}) is formed predominantly at the surface of Ti in the fluorine-containing solution. It is known that the TiO_2 nanotube pore formation is based on both electrical field assisted dissolution of the TiO_2 layer and the chemical dissolution by the fluorine-containing electrolyte. Both dissolution reactions occur at the same time and play a critical role in understanding the formation of TiO_2 nanotubes at the Ti surface.[16,17] When the Ti surface is first etched by the electrolyte solution, very small pits (sub-nanometer scale) are formed on the Ti surface and are rapidly restored to a TiO_2 layer. With continuous corrosion of the Ti surface by HF ions, the pits become nanoscale pores. As reaction time goes by, nanoscale pores become the main body of nanotubes, and the small pits which are still constantly formed at the latter stage of the processing become the interspaces between nanotubes. Another proposed mechanistic model further explains that prior to pit formation, there is an occurrence of microcracks in the TiO_2 layer, which further develop and guide the formation of the pits.[16] The anodization process and the TiO_2 nanotube structure fabricated by such a process are shown in Figure 1. The schematics in Figure 1a illustrate the electrochemical anodization apparatus, basic step-by-step, flow-chart procedure. The TiO_2 nanotube structure is often annealed at ~500°C to convert the amorphous nanotubes in the as-anodized condition to the desirable, predominantly anatase crystal structure. The microstructure of the TiO_2 nanotube arrays are presented by scanning electron microscopy (SEM) images (Figure 1b and c), and a cross-sectional transmission electron microscopy (TEM) image (Figure 1d).

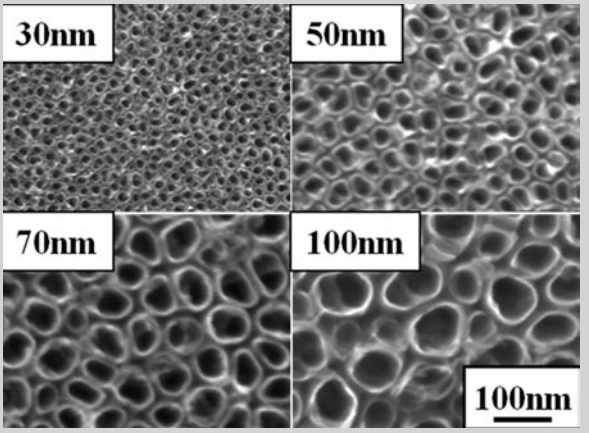

Figure 2. Comparative SEM micrographs of self-assembled TiO_2 nanotubes with different diameters. Highly ordered nanotubes with four different pore sizes between 30–100 nm are shown.

Based on the mechanism of nanotube formation, it is inherent that the nanotubular structure formation depends on both the intensity of applied voltage and the concentration of fluorine ions in solution. It is known that by increasing the applied voltage, larger diameter nanotubes can be formed. We will further discuss the effects of the diameter pore size by changing the voltage during anodization for optimizing the nanotube size for specific cell function and fate.

BIOMEDICAL APPLICATIONS

Surface TiO_2 Nanotubes as Orthopedic Implant Materials

For orthopedic and dental bone implants, the most common and successful biomaterial being used is Ti or Ti-base alloy (such as Ti-6%Al-4%V), which does not elicit an inflammatory response in vivo. The bone bonding generally occurs in a favorable manner between the implant metal and the underlying bone surface, without the common connective tissue layer that forms from the body's immune response (foreign body reaction).[18–20] However, a significant number of failures of orthopedic implants occur over many years of service, such as bone loosening, which forces undesirable re-surgery operations for patients. Roughened or nanostructured Ti and TiO_2 nanotube surfaces seem to obtain their osseointegration (the direct structural and functional connection between living

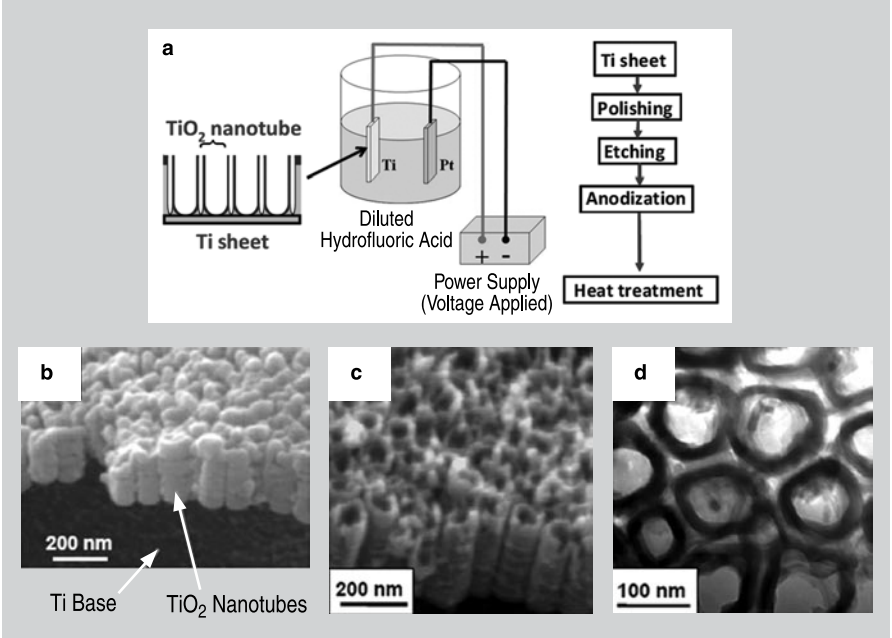

Figure 1. TiO_2 nanotube fabrication. (a) Schematic illustration of anodization process, (b) SEM image showing vertically aligned TiO_2 nanotube arrays on a titanium sheet, (c) higher magnification SEM image, (d) cross-sectional TEM image.

bone and implant) surface properties through mechanical interlocking. We have previously shown that heat-treated TiO_2 nanotube surfaces having a diameter and height approximately 100 nm and 300 nm, respectively, elicit anchoring sites for osteoblast (bone building cells) to adhere and grow directly into the nanotubes allowing for significantly up-regulated alkaline phosphatase activity, accelerated proliferation, and increased mineralization.[1] An important aspect of the TiO_2 nanotube system is that the nanotopography can feature a more defined, reproducible, natural, and reliable roughness than micro and macro-topography for enhanced bone cell function. It is also interesting to note (Figure 1d) that there are ~10 nm spaces between the nanotube walls[21] which, even after the cell adhesion, can allow for continued fluid flow of culture media and increased exchange spaces for gas, nutrients, and cell signaling molecules for an overall enhanced cell environment.

Effect of Nanotube Diameter on In Vitro Osteoblast Response

With a strong osseointegration capability demonstrated,[1] TiO_2 nanotube structures offer encouraging implications for the development and optimization of novel orthopedics-related treatments with precise control toward desired cell and bone growth behavior. We further investigated the in vitro behavior of osteoblasts (bone building cells) cultured on various inner pore (30–100 nm) diameters of vertically aligned TiO_2 nanotubes and investigated the nano-size effect on osteoblast cell adhesion, morphology and osteogenic functionality. A unique variation in cell behavior even within such a narrow range of nanotube dimensions was observed. We have obtained different diameters for the TiO_2 nanotube structure (30, 50, 70, and 100 nm diameter) by varying the anodization process parameters as shown in Figure 2. The images show highly ordered, vertically aligned nanotubes with four different pore sizes between 30–100 nm, created by controlling the applied voltage potentials ranging from 5 to 20 V during the anodization fabrication processing. The nanotubes differ in diameter and height proportionally with a diameter to height ratio of 1:3. The nanotube surface on the anodized Ti substrate is robust and clear. For the purposes of our studies, the maximum size of the TiO_2 nanotube diameter was limited to 100 nm in order to unify the experimental conditions and composition of electrolyte solution. The nanotopography significantly enhanced the roughness of the surface with the average roughness values (R_a) of ~13 nm and increased the hydrophilic surface characteristics showing contact angles less than 11°, while flat Ti metal without a nanostructure is less rough and more hydrophobic in nature with contact angle of ~54°.[22]

When comparing the effect of different diameter of TiO_2 nanotubes, it was found that there were distinct size regimes for controlling precisely the cell behaviors of initial adhesion and growth vs. elongation and increased alkaline phosphatase (ALP) activity of osteoblasts. In terms of cell adhesion and morphology, adhesion was the greatest on the smallest 30 nm diameter nanotubes over all other larger sizes of nanotubes, but the nanotube surfaces with a lower density of cells started to show an increase in morphological elongation with increasing nanotube diameters as shown in Figure 3a. The trend of ALP activity seemed to correspond with the elongation trend where ALP activity increased with increasing diameters reaching a peak on the largest 100 nm diameter TiO_2 nanotube surfaces as displayed in Figure 3b. It appears that 100 nm TiO_2 nanotubes, having the most increased biochemical ALP activity of osteoblast cells, hold the most promise for the greatest integration of orthopedic implant material into surrounding bone.

Furthermore, it has been suggested that the differences in cell responses can be explained by the presence of different curvature in the pores providing optimum compression and tension of cell mechanoreceptors.[23,24] It was also reported that the basement cell membrane specifically in contact with a nanostruc-

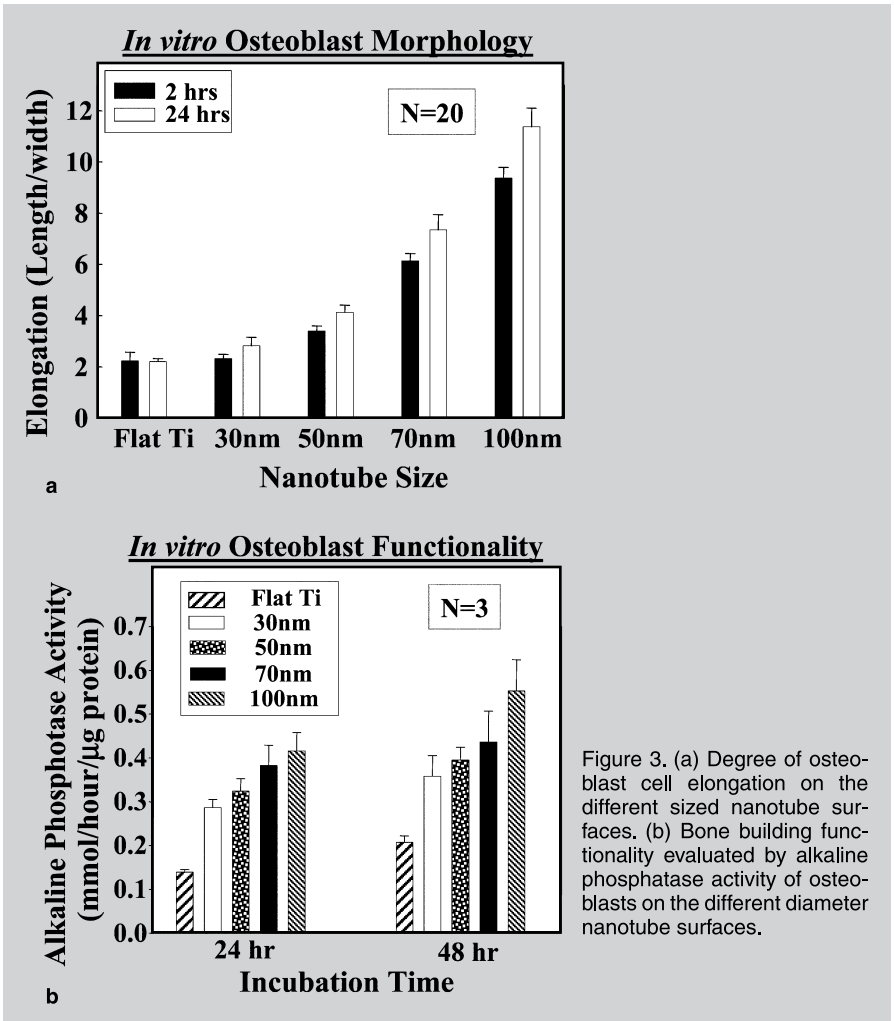

Figure 3. (a) Degree of osteoblast cell elongation on the different sized nanotube surfaces. (b) Bone building functionality evaluated by alkaline phosphatase activity of osteoblasts on the different diameter nanotube surfaces.

tured surface will suffer tensile and relaxation mechanical forces that will rearrange its components and/or open ion channels that will trigger cell behavior.[25] This may help explain the difference in osteoblast cellular responses by the different nanotube dimensions.

In Vivo Osseointegration in Rabbit Tibia Enhanced by Nanotube Surface

Under in vivo conditions, osteoblast cells adhere, proliferate, and mineralize regions of bone in direct contact with the orthopedic Ti implant. Instead of inserting bio-implant materials for the mechanical support of the bones, a more desirable goal is to produce osteoinductive materials that integrate directly into the bone. To evaluate the in vivo animal response to the TiO_2 nanotube surface (100 nm diameter) vs. conventional sandblasted Ti surfaces, five lop-eared rabbits were used in our studies. The details of the in vivo experimental procedures are given in a previous publication.[26] Briefly, two planar surfaces on each rabbit tibia were prepared with a 5-mm diameter three-prong router during saline cooling. The Ti sandblasted disks or TiO_2 nanotubes were implanted according to a rotational scheme to ensure that any observed difference was due to the implant surfaces, rather than the implant positioning. Teflon caps were placed over the implant surfaces not in contact with the bone to prevent bone overgrowth. Figure 4 clearly indicates a direct growth of new bone onto the nanotube surface with no trapped amorphous tissue layer at the implant-growing bone interface. In contrast, the sandblasted Ti implant shows a new bone-implant interface with a trapped soft tissue layer, which contributes to the well-known bone-loosening problem and implant failure at the bone-implant interface. As well, mechanical pullout tests proved that the implant/bone bond with the nanotube surfaces was ~5–9 times stronger than the conventional sandblasted implant surfaces. Noticeably, nanotube surfaces exhibited greater bone formation and bone-implant contact compared with sandblasted surfaces. Comparative SEM energy dispersive x-ray analysis (EDX) mapping of the implant-bone interface after tensile testing exhibited strong signals for the component of bone, i.e., the presence of calcium and phosphorus on the fractured interface of the TiO_2 nanotube surface, but not on the Ti sandblasted implant surface where only sporadic small regions showed calcium and phosphorus (Figure 4). These data indicate that the interface bonding with TiO_2 nanotubes is so strong that the fracture actually occurs within the new growing bone, rather than at the implant-bone interface. Calcium and phosphorus, which are indicative of strong osseointegration, covered 41.7% of the nanotube implant surface area compared to 8.3% for the sandblasted surface.

Effect of Nanotube Size on Mesenchymal Stem Cell Osteogenic Differentiation

Nanotopography in Stem Cell Research

A more recent and advanced concept for further improved orthopedics technology is to introduce a combination of nanotechnology and stem cell treatment. Mesenchymal stem cells (MSCs) are pluripotent adult stem cells available primarily from bone marrow, and are relatively easily obtained unlike embryonic stem cells. To produce the desired bone cells (osteoblasts), the MSCs need to be guided to selectively differentiate to osteoblasts, rather than differentiating into other types of cells such as myocytes, beta-pancreatic islet cells, adipocytes, or neural cells.

In recent years, nanotopography has been proven to have significant and favorable effects on stem cell commitment into mature lineages. In terms of bone cell (osteoblast) differentiation,

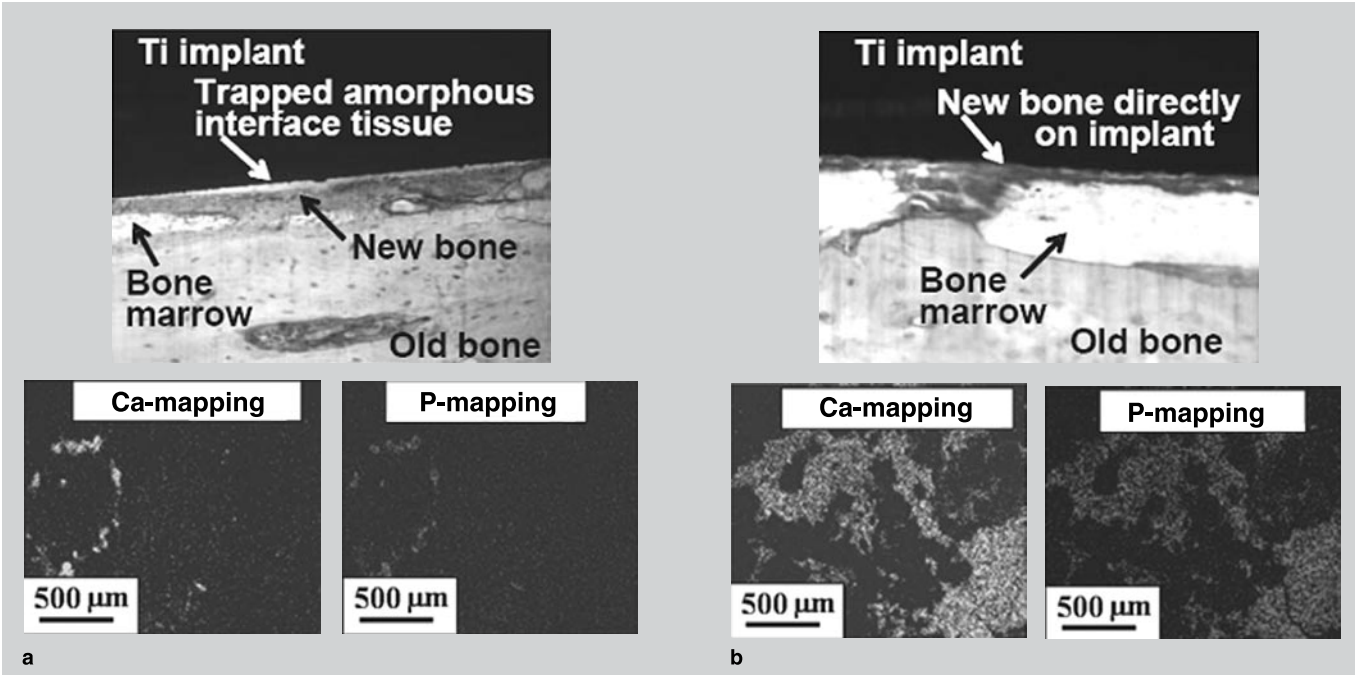

Figure 4. Histology studies showing comparative osseointegration at the implant surface of (a) standard sandblasted titanium, (b) TiO_2 nanotube surface implant. Cross-sectional microscopy of implant-new bone interface (upper images) and bone mineralization calcium and phosphorous mapping (lower images) on sandblasted titanium implant vs. TiO_2 nanotube surface implant are presented.

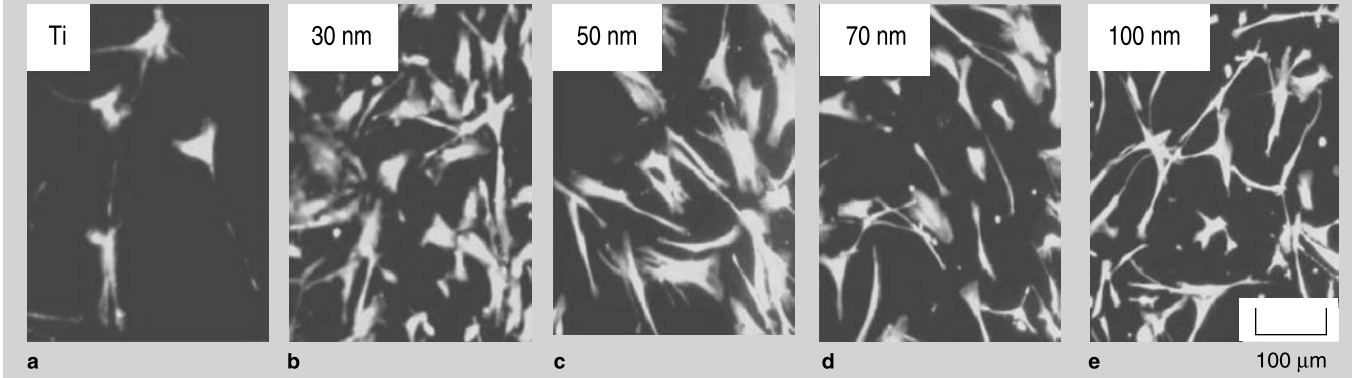

Figure 5. Mesenchymal stem cell (MSC) response to different sized (30, 50, 70, 100 nm diameter) nanotube surfaces with morphological observation of cells by FDA live staining. MSCs increase in elongation with increasing nanotube diameter.

Dalby et al.[10–12] have shown that random circular nanostructures promote and direct osteoblast differentiation of MSCs. This finding shows that the effect of nanoscale geometries alone can induce differentiation. These studies have demonstrated that different degrees of nanosymmetry or nanodisorder induces change in cell adhesion formation, which impacts on cytoskeletal tension, affects indirect mechanotransduction pathways and imposes morphological changes on cells. The surface nanotopography directly induces pronounced changes of cell shape, and consequently gene expression, which can potentially mediate differentiation of stem cells into various cell types.

The cell morphology/spreading tends to dominate the stem cell fate. McBeath et al. showed that commitment of stem cell differentiation to specific lineages is dependent upon cell shape.[27] In a single cell experiment with micropatterned surfaces the critical role of cell spread/shape in regulating cell fate was determined. As well, a recent study showed MSCs in multi-cell islands differentiated into different lineages depending on the MSC location in population. Cells located at the edges of the island exhibit osteogenic characteristics, whereas cells located in the center are adipogenic.[28] These findings have been interpreted on the basis that cells at the edges of the micropatterns experience higher tension (higher stress) than the cells at the center. The cellular tension/stress due to morphological shape control may provide the mechanical cues for controlling cell fate. Investigations with the manipulation of cell substrate rigidities have led to the same conclusion: MSCs on rigid surfaces (high tension) differentiated into osteogenic lineage, whereas MSCs on soft surfaces expressed neuron markers.[29] All these recent evidences clearly demonstrated that the MSC fate is determined by their responses to the physical nature of the substrate.

Through the interdisciplinary combination of materials science, nanotechnology, cell biology, and bioengineering to guide stem cell osteogenesis in vitro and in vivo, the goal of cell-based therapy for bone repair can be achieved. The realization of the full potential of MSCs in regenerative medicine requires selective differentiation. Our recent studies on nanotechnology substrates for control of osteogenic-related MSCs have shown the effects of nanotopography on specific differentiation by using only the geometric cues of the surface.[30] On substrates made of TiO_2 nanotube surface structures, we induced selective osteoblast differentiation by creating stem cell elongation accompanied by increased cytoskeletal stress using large dimensions of nanotube diameters (~100 nm). It is hypothesized that different nanotube diameters control MSC fate by having different effects on the cell morphology and consequently cytoskeletal stresses which in turn modulates differentiation.

Stem Cell Elongation and Osteogenesis on TiO_2 Nanotubes

While the major effort on controlling the fate of stem cells has been concentrated on biological or chemical means by many researchers, we have recently reported that a physical science approach alone can also induce the desired type of stem cell differentiation. The key point of this novel discovery is that the exposure of MSCs to substrates containing TiO_2 nanotubes with various diameters (Figure 2) can induce differential fates. For TiO_2 nanotubes with a small diameter such as ~30 nm, the stem cell differentiation was suppressed while adhesion and growth was accelerated. Such cell enrichment and proliferation without differentiation is a beneficial application of stem cell technology to cell therapy. In contrast, when the MSCs were exposed to a larger diameter nanotube substrate such as ~100 nm, the cells were significantly stretched with ~tenfold elongation as observed by SEM analysis and by live cell imaging using FDA (fluorescein diacetate) staining shown in Figure 5. We reported also a similar type of morphological effect by altered nanotube dimension on osteoblast cells behavior.[3]

The osteo-differentiation of these stretched MSCs was quantitatively determined by PCR (Figure 6b) and immunostaining of the osteogenic markers, alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN) (data not shown). Uniquely, MSCs on the largest 100 nm diameter TiO_2 nanotube surfaces had the highest up-regulation of the osteogenic RNA transcription levels as shown in Figure 6b. The 100 nm diameter samples were the only experimental samples that stained positively for OPN and OCN markers after two weeks of culture. The stem cell elongation and osteo-differentiation trends seem to correlate as shown in Figures 5 and 6. The suggested mechanism is in agreement with the general notion that the morphological elongation induced increased cytoskeletal stress which in

turn modulated differentiation into the specific osteoblast lineage. The overall results indicated that the MSC adhesion was facilitated by small-diameter nanotubes, while their osteogenic differentiation was facilitated by large-diameter nanotubes. Thus we have developed a concept of determining stem cell fate based solely on the geometric cues of the surface nanostructure.

CONCLUSION

Vertically aligned TiO_2 nanotubes exhibited critical and beneficial biological effects especially in the area of orthopedic implants. The adhesion and mineralization of osteoblasts were enhanced by the presence of TiO_2 nanotubes for significantly accelerated bone healing and mechanical interlocking both in vitro and in vivo. It has also been found that mesenchymal stem cell osteo-differentiation can be controlled by the physical dimension of the TiO_2 nanotube diameter for advanced therapies utilizing nanotechnology. The unique nanotopographical features and high-quality biocompatibility of the TiO_2 nanotube surface elicits many possibilities for tissue engineering and other biomedical applications.

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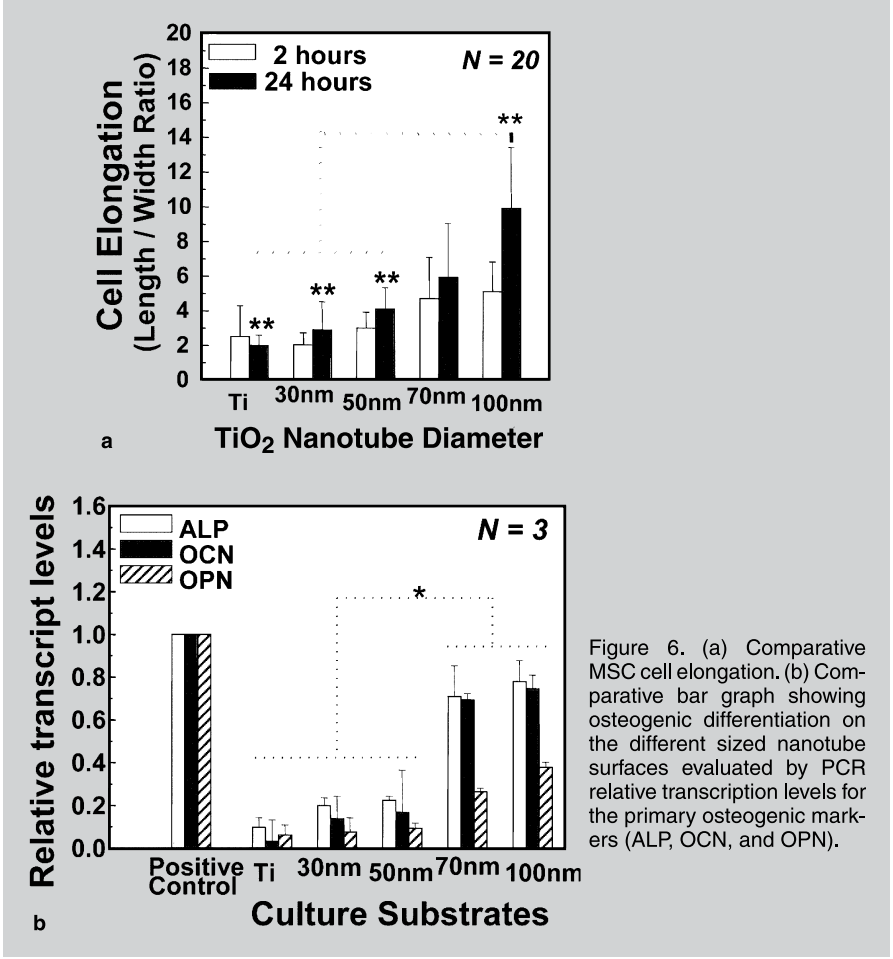

Figure 6. (a) Comparative MSC cell elongation. (b) Comparative bar graph showing osteogenic differentiation on the different sized nanotube surfaces evaluated by PCR relative transcription levels for the primary osteogenic markers (ALP, OCN, and OPN).

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