Effect of Nanoporous Alumina on the Growth Behavior of MG63 Osteoblast-like Cells and Human Mesenchymal Stem Cells （ナノポーラスアルミナによるMG63骨芽細胞様細胞および間葉系幹細胞の成長挙動に及ぼす影響）

宋　　遠会

Bone tissue is a type of dense connective tissue which gives the bones the strength that is required to act as levers for muscles, to give form to the soft tissues of the body, and to provide protective cavities for the vital organs. However, due to the high force impact or stress, or trivial injury such as osteoporosis, bone cancer, or osteogenesis imperfect, bone fractures are frequently happened in our daily life. By now, the repair of fractured bones is a serious challenge for clinician.

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes which restore, maintain, or improve tissue function or replace a whole organ. Compared with traditional artificial implants, tissue engineering aims at making a long-term functional biological replacement. The fundamentals of tissue engineering involve the cell sources and scaffolds for cell expansion, differentiation, and growth factors. Cells are cultured in a three dimensional scaffold with medium containing various soluble factors. After a tissue is developed in vitro, it needs to be implanted in vivo. With the advancement of tissue engineering, the development of cells based transplanted scaffolds provides a new insight for orthopedic treatment of bone fracture. An ideal bone tissue engineered scaffold are typically made of porous degradable materials which provide the mechanical support during the repair and regeneration of damaged or diseased bone. Furthermore, materials should preferably be osteoinductive (i.e. they are capable of promoting the differentiation of progenitor cells into osteoblastic lineage), osteoconductive (i.e. they can support bone growth and encourage the ingrowth of surrounding bone), and capable of osseointegration (i.e. they can integrate themselves into surrounding bone). Due to its excellent biocompatibility, mechanical properties and well established fabrication process, nanoporous alumina has raised a great attention. Selection of a reliable cell source is the next step after the development of an adequate porous

structure. In fact, an ideal cell source should be easily expanded to higher passage and non-immunogenicity and have a protein expression pattern similar to the tissue to be regenerated. Due to their non-immunogenicity, osteoblasts is the first, and most obvious choice of cell source, which can be isolated from the patients (autologous cells) and then followed by limited expansion in vitro. However, several limitations are shown in this methodology: few cells can be isolated from the tissue and their expansion rates are relatively low, which limit the number of available cells to be seeded on the scaffolds. Moreover, in some diseased bones, osteoblasts are not appropriate for transplantation because their protein expression profile is lower than the expected values. An alternative methodology is the use of cells obtained from xenogenous donors, which would solve the problem of low cell number. However, issues including the immunogenicity, the possibilities of the transmission of infectious virus and the ethical and social problems refrain the application of this approach. Since the first discovered by Friedenstein and his colleagues in 1976 from bone marrow, mesenchymal stem cells (MSCs) have attracted much attention as an attractive tool for tissue engineering applications. MSCs can easily be culture expanded to large numbers and have been reported to reach up to 50 population doublings in vitro. MSCs have a high capacity that with increased number of passages they did not spontaneously differentiate. Under certain circumstance, MSCs have been shown to be capable of differentiating into multiple cell types including adipocytes, chondrocytes, osteocytes, and cardiomyocytes. Furthermore, it has been suggested that these cells may possess immunosuppressive effects which may render them either immune privileged or perhaps being immunosuppressive roles in vivo, which would make them suitable for allogeneic or xenogeneic transplantation. Easy to isolation, higher proliferative capacity, multiple differentiation potential, immunological compatibility and no ethical conflict made MSCs being an attractive cell source for tissue engineering application. Differentiation of stem cells is affected by many factors. Various growth factors and steroids play important roles in regulating bone development and bone remodeling. Reports showed that MSCs obtained from young adult rats can differentiate into osteoblasts and express bone like structure when cultured with α-glycerophosphate and dexamethasone. Bone morphogenetic proteins (BMPs) belong to the transforming growth factor beta (TGF-β) superfamily. This group of proteins affect proliferation of osteoblasts and cause increased matrix synthesis by osteoblasts. Previous studies have proved that BMPs were able to induce the differentiation of MSCs into osteoblasts.

This dissertation aims to study the effect of nanoporous alumina on the growth behavior of MG63 osteoblast-like cells and MSCs. The nanoporous alumina was selected as tissue engineered scaffold for cell culture. In addition, the surface of the nanoporous alumina was functionalized with BMP2 and the synergetic effect of BMP2 and substrate topography on the proliferation and differentiation of MSCs were investigated. Furthermore, nanoporous alumina was used as drug delivery device to store BMP2 and the effect of the controlled released BMP2 and surface topography on the proliferation, migration and osteogenic dif-

ferentiation of MSCs were studied.

Chapter 1 is a general introduction of the research background, such as the repair of bone defects, the development of tissue engineering, as well as a review of the importance, usefulness, and significance of nanoporous alumina, MSCs and BMP2 in the bone tissue regeneration.

Chapter 2 introduces the materials and methods used in this study. Nanoporous alumina substrates with different pore sizes, MG63 cells and MSCs were employed in the study. BMP2 was used to induce the osteogenic differentiation of MSCs. Experimental methods including cell viability assay, immunofluorescence staining, real-time PCR, wound healing assay, alkaline phosphatase (ALP) activity and mineralization assay were described.

Chapter 3 represents the growth behavior of MG63 osteoblast-like cells on nanoporous alumina substrates with different pore sizes. MG63 cells were cultured on cover glass and nanoporous alumina surface with the pore size of 20, 100 and 200 nm to investigate the interplay between surface topographies of nanoporous alumina and cellular behaviors, such as cell viability, cell morphological change. Moreover, the differentiation of MG63 cells on different substrates were also detected. Results showed that cell proliferation and differentiation are strongly correlated with the alumina pore size. Reduced cell viability and integrin β 1 expression, increased cell elongation and prominent filopodia were documented for cells cultured on alumina surfaces with the larger pore sizes. Moreover, MG63 cells cultured on nanoporous alumina with the larger pore sizes also exhibited higher osteoblast differentiation markers, such as ALP activity and extracellular matrix mineralization.

Chapter 4 describes the in vitro proliferation and differentiation of MSCs on nanoporous alumina. MSCs were cultured on smooth alumina and nanoporous alumina substrates with the pore size of 20 and 100 nm, respectively, and the size effect of nanoporous alumina on MSCs growth behavior in terms of proliferation, morphology, expression of integrin β 1 were evaluated. Osteogenic differentiation was also evaluated by detecting the ALP activity, osteocalcin expression and extracellular matrix mineralization in the presence of induction medium. It is found that cell adhesion, proliferation and differentiation are strongly regulated by the alumina pore size. With the increasing pore size of nanoporous alumina, reduced cell numbers, increased cell elongation and prominent filopodia were detected. Moreover, the expression of osteogenic differentiation markers including ALP activity, osteocalcin expression and extracellular matrix mineralization also increased with the increasing pore size of alumina, and MSCs cultured on nanoporous alumina with 100 nm pores exhibited the highest osteoblast differentiation in the presence of induction medium.

Chapter 5 presents the surface functionalization of nanoporous alumina with BMP2 and its

synergetic effect on the differentiation of MSCs. By introducing polydopamine as covalent coupling reagent, BMP2 was immobilized onto the surface of alumina substrates. MSCs were cultured on different substrates and the synergistic effect of BMP2 and substrate topography on proliferation and differentiation of MSCs was investigated. Scanning electron microscopy and X-ray photoelectron spectroscopy (XPS) results proved that BMP2 was successfully conjugated onto the alumina substrates. Compared with untreated alumina substrates, BMP2 immobilized alumina substrates were able to promote adhesion and spreading of MSCs. Moreover, surface functionalized alumina substrates promoted the expression of integrin β 1. Significantly higher ALP activity and extracellular matrix mineralization were detected in cells cultured on BMP2 immobilized alumina substrates compared to those cultured on untreated substrates. The results suggested that surface functionalization of nanoporous alumina substrate with BMP2 was beneficial for cell growth and osteogenic differentiation.

Chapter 6 shows the application of nanoporous alumina as nanoreserviors to store and control the release of BMP2 for inducing the differentiation of MSCs. After the BMP2 was loaded in the nanoporous alumina, chitosan was employed to seal the nanopores by spin coating. With the degradation of chitosan, BMP2 was released from the nanopores. The effect of released BMP2, together with nanoporous alumina substrates, on cell migration and osteogenic differentiation of MSCs were evaluated. By encapsulating the BMP2 loaded nanoporous alumina with chitosan films, the bioactive and release rate of BMP2 were well controlled. Cell viability assay, morphological observation and wound healing assay indicated that BMP2 was advantageous for cell growth. The results of in vitro tests confirmed that BMP2 loaded alumina substrate could promote osteoblastic differentiation of MSCs.

Chapter 7 is the conclusion of this thesis. It is found that cell adhesion, proliferation and differentiation of MSCs are affected by the surface topography of nanoporous alumina. In the presence of BMP2, MSCs could be induced into osteoblast by the synergistic effect of BMP2 and substrate topography. The results indicate that nanoporous alumina can be used as scaffold in bone tissue engineering.

The beneficial effects of the surface topography and BMP2 on cellular behavior presented in this dissertation suggest that this novel biocompatible material, used in combination with stem cells and growth factors, can serve as an effective bone tissue engineered scaffold in therapeutic and regenerative medicine.