Micro- to Nano-patterned Titanium Improves and Guides *In Vitro* Adhesion of Bone Marrow Stromal Cells

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Statement of Purpose: The long-term efficacy of titanium (Ti) implants for orthopedic applications relies greatly on the ability of the implant to elicit implant-tissue integration, namely osseointegration (Liu H. J Biomed Mat Res A. 2011; 99A: 249-60). Improved osseointegration of load-bearing Ti implants can potentially help address and reduce revision surgeries that result from: (i) implant loosening caused by a combination of cyclic loading and lack of juxtaposed bone formation (Riedel NA. J Mat Sci. 2011; 46: 6087-95); and (ii) generation of harmful wear particulates that may result in osteolysis (Agarwal S. Curr Orthopaedics. 2004; 18: 220-231). Previous studies have shown that surface microstructure and topography are closely related to bone growth, as indicated by improved osteoblast activity (Liu H. Nanotechnology. 2005; 16: S601-8). Bone marrow stromal cells (BMSCs) provide vertebrates the continuous supply of osteoblasts needed for bone remodeling and repair (Rickard DJ. Dev Biol. 1994; 161: 218-28), thus motivating their use in this *in vitro* study to evaluate cell adhesion and growth on micro- to nano-patterned Ti substrates at 24 and 72 hr of cell culture.

Methods: Uniform, high precision surface patterns were defined on bulk Ti substrates using a novel micromachining technique. Briefly, polished Grade 1 Commercially Pure Ti substrates (99.6% Ti, Tokyo Stainless Grinding Co., Ltd, Japan; RA~ 10 nm RMS) were ultrasonically cleaned, dried with N_2, and dehydrated at 150°C. They were then primed with hexamethyldisilazane, spin-coated with photoresist, and lithographically patterned. Each 17 x 17 mm^2 substrate contained nine 5 x 5 mm^2 sub-patterns, each composed of a periodic array of grooves with widths ranging from 0.5 µm to 50 µm, and each pitch twice the groove width. The patterns were then transferred into the Ti substrate to a depth of 1.3 µm using the titanium inductively coupled plasma deep etch (TIDE) process, which is a highly-selective and anisotropic Cl_2/Ar dry etch (Parker ER. J Electrochem Soc 2005; 152: C675–83). Following ultraviolet exposure and autoclaving of the samples, BMSCs harvested from rat weanlings were seeded directly onto the patterned substrates at a density of 50,000 cells/cm^2. The substrates were then incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) under standard cell culture conditions for 24 and 72 hr. At the conclusion of each prescribed time interval, non-adherent cells were removed by washing with PBS. Adherent cells were fixed with 4% formaldehyde (10% neutral buffered formalin) and stained with 4', 6-diamidino-2-phenylindole dilactate (DAPI) nucleic acid stain and Alexa Flour® 488 cytoskeleton stain. Adherent cells on each sub-pattern were visualized and counted based on the fluorescence of DAPI-stained nuclei using a fluorescence microscope (Nikon Eclipse Ti). Numerical data sets were evaluated for statistical relevance using the ANOVA and Kruskal-Wallis tests followed by standard post-hoc tests; statistical significance was considered at $p<0.05$.

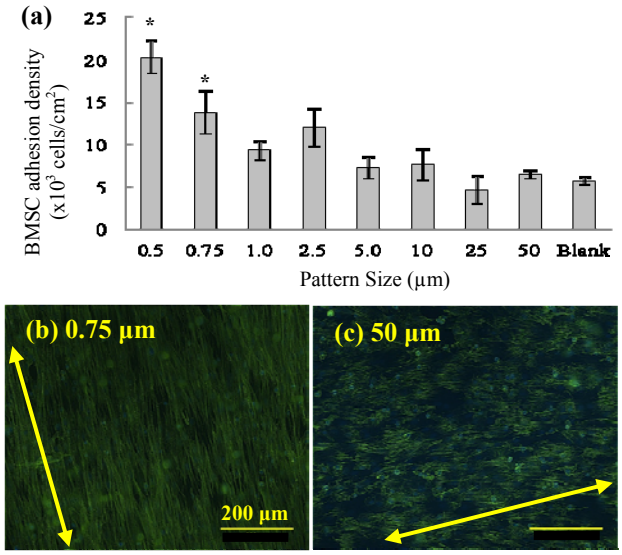

Figure 1: (a) BMSC adhesion density on titanium patterned surfaces and on unmodified titanium surface (blank) used as reference at 24 hr of cell culture (mean ± SEM; n=6; *$p<0.05$ compared to blank sample). Fluorescence images of BMSC adhesion and growth at 72 hr of cell culture on (b) 0.75 µm, and (c) 50 µm Ti micropatterns (scale bars = 200 µm).

Results: Increasing BMSC adhesion density was observed with decreasing pattern feature size after 24 hr of cell culture, as seen in Figure 1a. Specifically, significantly higher adhesion density was observed only on the 0.5 µm and 0.75 µm patterns when compared with the non-patterned Ti (blank). Fluorescence images from the 72 hr culture showed higher cell adhesion and preferential alignment on the smaller feature size patterns. For example, the 0.75 µm (Figure 1b) pattern showed higher adhesion and preferential growth along the direction of the pattern (shown as double arrows) when compared with the larger 50 µm patterns (Figure 1c), which showed less identifiable cellular alignment.

Conclusions: Uniform and high precision patterned Ti surfaces with features ranging from 0.5 µm to 50 µm were used to promote BMSC adhesion and preferential growth on Ti. Results from 24 hr and 72 hr *in vitro* cultures indicated improved BMSC adhesion and preferential growth along the direction of the patterns on the 0.5 µm and 0.75 µm patterns. Future work with specific bio-markers to evaluate other cellular activities, such as differentiation and proliferation, are necessary to understand BMSC response to patterns of different sizes and identify those that yield optimal results for orthopedic implant applications.