Surface-Mediated Immunomodulation of Osteogenic, Inflammatory, and Angiogenic Factors Produced by Human Bone Marrow Stromal Cells

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INTRODUCTION: Previous studies investigating bone-implant surface modifications have predominantly focused on optimizing their osteogenic capacity. However, successful implant osteointegration requires a favorable immune microenvironment to coordinate the numerous immune cell types, signaling molecules, and cytokines responsible for bone-related pathophysiological processes. Thus, to improve implant success *in vivo* it is critical to define not only the osteogenic properties of implants, but the immunomodulatory effects exerted within the peri-implant environment. The purpose of this study was to use a customized inflammasome and angiogenesis specific qPCR array to evaluate the differential expression of 100 genes by human bone marrow stromal cells (MSCs) to compare the immunomodulatory, osteogenic, and angiogenic responses to different surface modifications. A customized RT-qPCR gene array was developed to evaluate the osteogenesis-relevant expression of inflammatory and angiogenic mediators produced in response to different implant surface modifications.
METHODS: MSCs were cultured for 14 days on tissue culture polystyrene (TCPS), polyether-ether ketone (PEEK), or titanium-aluminum-vanadium (Ti6Al4V) discs with different surface modifications (anodized [Anodized], machined smooth [Smooth], and micro/nanoscale [MN] surface technology). Briefly, confluent human female MSCs were plated on surfaces at 20,000 cells/well in 24-well plates. 12 discs were used per variable and wells were pooled in duplicate (n=6) to generate sufficient mRNA for qPCR analysis. On day 14, cultures were incubated with fresh media for 24 hours. Total RNA was extracted in TRIzol and gDNA removed by phenol-chloroform extraction, ethanol precipitation, and treatment with DNase I. Reverse transcription and qPCR were performed using the High-Capacity cDNA RT Kit and Power SYBR Green Master Mix (Applied Biosystems) with optimized gene-specific primers designed in-house. The results were analyzed using the $2^{-\Delta\Delta Ct}$ method to calculate the expression levels of 100 osteogenic, inflammasome and angiogenesis-related target genes and normalized to the reference genes, GAPDH and PPIA. Comparisons were made between test surfaces by one-way ANOVA for multiple comparisons with Bonferroni correction. Statistical significance was determined at $p \leq 0.05$.

RESULTS: MSCs cultured on MN-textured surfaces exhibited robust osteogenic effects, with significantly enhanced expression of genes encoding the osteoblast marker, osteocalcin (BGLAP), as well as factors responsible for modulating osteoblastic differentiation, bone morphogenic protein 2 (BMP2) **(Fig. 1A)**, and Runx-related transcription factor 2 (RUNX2) **(Fig. 1B)**. The integrin expression profile of MSCs cultures on MN surfaces revealed significantly enhanced expression of integrin subunits α2 (ITGBA2) and β1 (ITGB1) **(Fig. 1C)** and reduced expression of integrin α5 (ITGA5). Integrin α6 (ITGA6), a MSC marker and mediator of endothelial cell proliferation and migration, was also upregulated **(Fig. 1D)**. MN surfaces promoted angiogenesis, stimulating an increased release of pro-angiogenic factors including vascular endothelial growth factor A (VEGFA) **(Fig. 1E)**, fibroblast growth factor 2 (FGF2), and connective tissue growth factor (CTGF), accompanied with significantly decreased expression of the endogenous angiogenesis inhibitor, semaphorin 3A (SEMA3A), compared with Smooth and Anodized surfaces. In addition, markers and effectors associated with pro-inflammatory M1 macrophage polarization (IL1α, IL1β, and IL-6) were significantly downregulated on MN surfaces compared to Smooth and Anodized surfaces **(Fig. 1F)**. Concurrently, MN stimulated substantially lower levels of neutrophil-recruiting monocyte chemoattractant protein-1 (MCP-1) **(Fig. 1G)** and the expression of Toll-like Receptor 4 (TLR4), a pattern recognition receptor involved in fibrous capsule formation, was downregulated in response to MN.
DISCUSSION: MN discs with combined micro/nano-scale surface features possessed superior pro-osteogenic and anti-inflammatory properties *in vitro* compared to TCPS and smoother, "machined" implants. MN surfaces exerted immunomodulatory effects on the osteoimmune microenvironment, enhancing the release of osteogenic and pro-angiogenic factors at the implant surface. In addition, increased VEGF expression and decreased levels of M1-specific cytokines indicate that MN surface texture may elicit M2 phenotypic switching of macrophages to an anti-inflammatory, pro-regenerative phenotype capable of suppressing inflammation, inducing angiogenesis, and promoting regeneration.
SIGNIFICANCE: Implant surfaces with combined micro/nano-scale surface features demonstrate favorable osteogenic and immunomodulatory properties for improving osseointegration, and represents an attractive strategy for improving long-term implant success *in* vivo, exerting their effects at the molecular, cellular, and tissue levels. However, mechanistic studies are still needed to understand the complex biomaterial-mediated immune responses that regulate bone regeneration**.**
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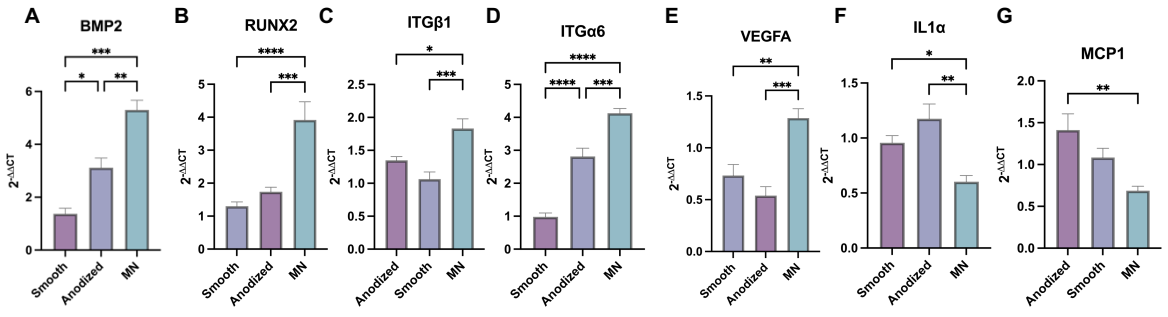

Figure 1. Relative gene expression of human MSCs cultured on Anodized, Smooth, or MN surfaces normalized to GAPDH.