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Osteogenic potential of human adipose-tissue derived mesenchymal stromal cells cultured on 3D-printed porous structured titanium

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Abstract

Integration of porous metal prosthetics, which restore form and function of irreversibly damaged joints, into remaining healthy bone is critical for implant success. We investigated the biological properties of adipose-tissue derived mesenchymal stromal/stem cells (AMSCs) and addressed their potential to alter the *in vitro* microenvironment of implants. We employed human AMSCs as a practical source for musculoskeletal applications, because these cells can be obtained in large quantities, are multipotent, and have trophic paracrine functions. AMSCs were cultured on surgical-grade porous titanium discs as a model for orthopedic implants. We monitored cell/substrate attachment, cell proliferation, multipotency, and differentiation phenotypes of AMSCs upon osteogenic induction. High-resolution scanning electron microscopy and histology revealed that AMSCs adhere to the porous metallic surface. Compared to standard tissue culture plastic, AMSCs grown in the porous titanium microenvironment showed differences in temporal expression for genes involved in cell cycle progression (*CCNB2*, *HIST2H4*), extracellular matrix production (*COL1A1*, *COL3A1*), mesenchymal lineage identity (*ACTA2*, *CD248*, *CD44*),

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osteoblastic transcription factors (*DLX3*, *DLX5*, *ID3*) and epigenetic regulators (*EZH1*, *EZH2*). We conclude that metal orthopedic implants can be effectively seeded with clinical-grade stem/stromal cells to create a pre-conditioned implant.

Graphical abstract

Keywords

mesenchymal stem cell; biomaterial; gene expression; extracellular matrix; scanning electron microscopy

INTRODUCTION

Prosthetic joint replacements improve patient mobility by mitigating arthritis-associated pain, traumatic injury, tumor resection, and genetic defects [1]. The occurrence of such procedures has increased dramatically: cumulative total hip arthroplasty and total knee arthroplasty procedures exceed more than 1 million per year in the US [2, 3], and are projected to increase 4-fold by the year 2030 [4, 5]. Even low rates of prosthetic joint failures produce a significant burden on health care systems [5], and failures related to poor osseointegration (i.e., bone growth into the porous implant surface) can lead to long term patient disability. Implant failure commonly leads to absent, damaged, or fractured local bone. These complications create a compelling need for rapid and successful osseointegration that increases implant durability and clinical performance.

To prevent prosthetic joint failures, implants are optimized for high strength-to-weight ratios, improving biological fixation [6, 7]. Advancements in metallic biomaterials include composing implants of multiple alloyed and composite metals, such as tantalum, titanium and chromium, and surface modifications that increase the surface area available for cell attachment. However, to meet the increasing demands of aging patients, additional strategies are required to improve outcomes for patients with poor bone quality and/or complex reconstructive requirements. As a corollary to advancements in orthopedic implant design, adult tissue-derived stromal/stem cells are being investigated, because such cells retain

trophic paracrine functions that may benefit the cellular microenvironment of healing tissues [8, 9]. Cell transplantations permit customization of implant components that may be further investigated using animal models of osseointegration [10].

To address the clinical need of improving implantation, the use of mesenchymal stromal/stem cells in adult reconstructive surgery merits additional exploration. Of particular interest are adipose-tissue derived mesenchymal stromal/stem cells (AMSCs); cells that can be harvested in large quantities while retaining their multi-lineage differentiation potential (e.g., into osteogenic, chondrogenic and adipogenic cells) [11]. According to Crespo-Diaz et al. [12], the yield of mesenchymal stromal cells per ml volume of adipose tissue is approximately 80,000-110,000, which is nearly 10-fold greater than an equivalent volume of bone marrow. For this investigation, we define AMSCs as mesenchymal progenitor cells of stromal origin (adipose tissue) with multi-lineage differentiation potential. However, the phenotype of proliferating AMSCs may change as cells become adapted to plastic-adherence and such phenotypic alterations may compromise their multi-lineage capabilities. Differentiation of AMSCs toward the osteoblast lineage, which results in extracellular matrix (ECM) production and mineralization, has been well-studied on tissue culture polystyrene (tc-PS) [12-14]. Therefore, a key question is whether AMSCs can be adapted to materials used for orthopedic implantation to generate a biologically favorable microenvironment for osseointegration.

Results presented in previous studies indicate that a number of mesenchymal and/or osteogenic cell types are capable of surviving, proliferating and differentiating on select metallic substrates [15-30]. In this study, we cultured clinical-grade AMSCs on surgical-grade porous structured titanium (ps-Ti) currently used in reconstructive joint arthroplasty. Our main findings are that: (i) ps-Ti is a suitable *in vitro* cell culture substrate for AMSCs, (ii) temporal expression of mRNAs for bone-related collagenous and non-collagenous ECM proteins upon osteogenic induction of AMSCs suggest positive changes to the implant microenvironment, and (iii) select histone modifying enzymes may act as suitable implant enhancement reagents due to their consistent patterns of regulation among cell donors.

METHODS

Porous structured titanium

ps-Ti discs (3 mm height; 25 mm diameter) comprised of alloyed titanium, aluminum, vanadium, and trace elements (Ti6Al4V) were provided by Stryker Orthopedics (Mahwah, NJ). The detailed fabrication method used in the manufacture of these discs, laser sintered additive manufacturing, has been described elsewhere [31]. Using an EOS 280 laser sintering machine, a powder of titanium alloy is melted by a computer-guided laser, which constitutes beads into solid form. This process is then continued layer-by-layer until a fully customizable, porous, three-dimensional metal shape is constructed. The resulting materials have the following parameters: strut size of $185.7 \pm 8.4 \mu\text{m}$, pore size of $408.6 \pm 89.5 \mu\text{m}$, overall porosity of $65.2 \pm 3.1 \%$, yield strength of $176.13 \pm 1.00 \text{ Mpa}$, an average modulus of elasticity of $3.48 \pm 0.26 \text{ GPa}$, and a random pore distribution [31]. The surface roughness of this material was $81.372 \pm 6.297 \mu\text{m}$, as measured at five independent points on one of the ps-Ti discs using a Zygo Newview white light interferometer (Zygo Corporation,

Middlefield, CT). The constructs used in this study were designed to mimic the *in vivo* architectural environment of trabecular bone. A vacuum furnace was used to relieve any residual stresses from the laser sintering, and a cleaning process of passivation and gamma sterilization was performed to establish functional equivalency to current medical devices that are similarly fabricated.

Mesenchymal cells from adipose tissue

Human AMSCs were isolated from adipose tissue samples (i.e., lipo-aspirates) obtained from three de-identified healthy donors with written individual consent and institutional approval (Mayo Clinic Institutional Review Board, Rochester, MN). These cells have been in routine use at our institute specifically for clinical trial applications and have been extensively tested for cell surface markers, RNAseq transcriptome profiles and multi-lineage potential [12-14]. Cell isolation and culture conditions have previously been described in detail [12, 32, 33]. In brief, cells are isolated following fat tissue digestion with 0.075% Type I collagenase (125 units per mg dry weight; Worthington Biochemicals, Lakewood, NJ) for 1.5 h at 37°C. Adipocytes, which are more buoyant than mesenchymal stromal cells, were removed by low speed centrifugation (400 g for 5 min). The resulting stromal vascular fraction was rinsed with phosphate buffered saline (PBS) and strained (70 µm cell strainer; BD Biosciences), while erythrocytes were eliminated by lysis (154 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). Mesenchymal stromal cells in the resulting fraction were expanded in Advanced MEM (Life Technologies, Grand Island, NY), 5% human platelet lysate (PLTMax; Mill Creek Life Sciences, Rochester, MN), 2 mM Glutamax (Life Technologies, Grand Island, NY), 2 U/ml heparin, 1% Penn-Strep (100 U/ml penicillin, 100 µg/ml streptomycin (Cellgro, Corning, NY)). Osteogenic supplements 50 µg/mL ascorbic acid (Sigma-Aldrich, St. Louis, MI), 10 mM β-glycerolphosphatase (Sigma-Aldrich, St. Louis, MI), and 392.46 µg/mL dexamethasone (Sigma-Aldrich, St. Louis, MI) were added to the base medium on day 0 of experimentation. Previously published studies have shown that AMSCs grown for extended culture periods without inductive stimulus may detach or spontaneously differentiate into cells with connective tissue properties [13]. The key comparison in our present study is the phenotype of cells at the start of the experiment when cells adhere to titanium and the final biological state at the end of the osteoblastic time course.

During passivation, cells were released from T175 plates using 3mL of trypLE Express dissociation reagent (Life Technologies, Grand Island, NY) for 3 minutes at 37° C, followed by the addition of 12 mL of maintenance medium to stop dissociation. The remaining solutions containing the cells were centrifuged at 1100 rpm for 5 minutes and aspirated to form a cell pellet. Pellets were suspended in 30 mL of fresh osteogenic medium and plated in T175 cell culture plates. All cells used in experimentation were of passage 6 or 7, which is comparable to clinically administered cell populations, as they are capable of differentiation at later passage levels [12].

Cell sampling strategy

Cells were cultured in 6-well polystyrene plates (Corning Inc., Corning, NY). Each ps-Ti well was coated with poly 2-hydroxyethyl methacrylate (Sigma-Aldrich, St Louis, MO) to

ensure growth of cells on the titanium discs and to prevent cell migration onto polystyrene. Standard two-dimensional tissue culture polystyrene (tc-PS) wells were used in parallel for comparison with ps-Ti experiments. To accomplish adsorption, cell suspensions of approximately 2.5×10^5 cells in 500 μL were applied to either the surface of ps-Ti discs (placed in the well of a 6-well plate) or tc-PS wells. An additional 500 μL of medium was added to the bottom of each well to prevent desiccation of the cells during adsorption. After 2-3 hours of adsorption in a standard incubator (37°C and 5% CO_2), 3 mL of media were added to each well (day -1). Following adsorption, the cells were incubated for 24 h, and maintenance medium was replaced with osteogenic medium (day 0). Osteogenic media changes were performed every 3-4 days (i.e., twice weekly) throughout the 28-day duration of the study (Table 1; Supplementary Figure 1).

RNA Isolation & RT-qPCR

At each time point (days 0, 3, 7, 14, 21, and 28), cells from ps-Ti discs and tc-PS were lysed using Qiazol and further processed with the Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA). Sampled RNA quantity (ng/ μL) and integrity (260/280 absorbance ratio) were measured using a Nanodrop 2000 (Thermo Fisher Scientific Inc, Minneapolis, MN). Reverse transcriptase-PCR reactions were performed in a total volume of 40 μL using the Superscript III RT kit (Invitrogen, Waltham, Massachusetts, USA.) Quantitative real-time PCR (RT-qPCR) reactions were prepared using the QuantiTect SYBR Green PCR kit (Qiagen) in a total volume of 10 μL with 0.05 μL of cDNA (10 ng/ μL). Primer sequences used in qPCR reactions were selected as appropriate based on preliminary RNA-seq analyses conducted by our research group, and are listed in Table 2. Specifically, *CCNB2* was selected as a marker of proliferation because it is specific for mitosis and therefore very clearly marks cells undergoing cell division, unlike cyclin D which is constitutively expressed in both cycling and non-cycling cells. Adiponectin was measured because the cells originate in adipose tissue, thereby making it important to determine the extent of differentiation toward the osteoblastic lineage. In our study, cells retain their adipose-tissue derived identity while becoming osteoblast-like.

Quantification measurements (C_t) were conducted using a CFX384 Real-time System c1000 Touch Thermal Cycler (BioRad, Hercules, California). Transcript levels measured by RT-qPCR were normalized to the housekeeping gene GAPDH using the 2^{-C_t} method. Additionally, the combined matrix of all mRNA expression values in multiple differentiation time-courses was log-transformed, row-centered and standardized to account for biological changes in GAPDH gene expression.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was conducted once on a naked ps-Ti disc (without cells), as well as cell-seeded ps-Ti discs at each time point (days 0, 3, 7, 14, 21, and 28). Discs were immersed in Trump's fixative (pH of ~ 7.3) to preserve cell attachment. The following treatments were performed to rinse, fix and desiccate each sample: (i) PBS (2x), (ii) water (2x); (iii) 10% ethanol; (iv) 30% ethanol; (v) 50% ethanol; (vi) 70% ethanol; (vii) 90% ethanol; (viii) 95% ethanol; (ix) 100% ethanol (2x). Discs were then placed in a critical point dryer while still in 100% ethanol and subsequently attached to an aluminum stub by

double-sided conductive tape. Final preparation included sputter-coating for 90 s with gold-palladium. In total, images at low (30x), medium (200x), and high (1300x) magnification were obtained for each sample (Table 1; Supplementary Figure 1).

Sectioning & Staining

To measure three-dimensional growth, AMSCs from a fourth donor were seeded onto ps-Ti discs for 14 days. To preserve the state of cells attached to the ps-Ti disc, they were rinsed in PBS, transferred to neutral buffered formalin (10%) for 24 hours and then placed in 70% ethanol. Each disc was then embedded in acrylic resin (methyl methacrylate monomer; Fisher Scientific) and cut into full-width sagittal sections 200 μ m thick using a diamond-tipped band saw (Exakt Technologies, Oklahoma City, OK). To highlight cell formation, two histological procedures, Sanderson's rapid bone stain (Dorn & Hart Microedge, Villa Park, IL) and modified Gomori's stain (Sigma-Aldrich, St. Louis, MO), were conducted on representative AMSC samples from day 14 (i.e., cell donor 258). Modified Gomori's staining involves soaking the slides in a mixture of Weigert's hematoxylin followed by a rinse with distilled water, a 20 minute soak with Gomori stain, a rinse with 1% acetic acid (2x), and dehydration. Stained images were captured using a Sony 3CCD DSP instrument. ImageJ, an open-source NIH image analysis software program, was used to evaluate three-dimensional AMSC integration (i.e. depth of penetration) into pores of the ps-Ti scaffold.

RESULTS

Orthopedic implant devices normally function to restore joint mobility and relieve pain, but some implants fail. Our goal was to coat ps-Ti with AMSCs and monitor their attachment to the ps-Ti substrate, proliferation and survival, as well as their ability to differentiate along the osteogenic lineage and initiate bone-related ECM production. This strategy is warranted because successfully combined application of implants and AMSCs may reduce the rate of primary arthroplasty failure and improve the outcomes of revision procedures.

Surface adherence and pore integration

AMSCs retain the intrinsic ability to become osteogenic upon induction with appropriate tissue culture cocktails [13]. Thus, as a possible strategy to improve implant osseointegration, we considered whether autogenic or allogeneic AMSCs are viable when seeded onto orthopedic devices prior to implantation. We first examined the attachment of cells to the horizontal and vertical surfaces of metallic discs. Throughout our studies, AMSCs were initially seeded in the presence of normal maintenance medium (day -1 to day 0). Cells were subsequently cultured in osteogenic medium to promote production of an osteoblast-like extracellular matrix and avoid spontaneous differentiation into less defined fibroblastic phenotypes, as previously noted [13]. ps-Ti has a complex three-dimensional structure with macro-, micro-, and nano-scale features (e.g., struts, pores, and beads; Fig. 1a) that are suitable for the adherence of AMSCs (Fig. 1b). Vertically, AMSCs migrated into the pores of ps-Ti discs, as evidenced by a cross-sectional analysis showing positive staining of cell growth and/or migration to a depth of approximately 1 mm after 14 days (Fig. 1c). Thus, ps-Ti discs support the three-dimensional growth of AMSCs.

Phenotypic variation in cell growth

Next, we examined whether osteogenic AMSCs from several different patients (Table 1) display the same adherence pattern. Based on SEM analysis, AMSCs exhibit patient-to-patient (or sample-to-sample) variation in cellular attachment and lateral expansion on ps-Ti discs. SEM images revealed the lateral expansion of proliferating cells and complete coverage of the ps-Ti substrate. This expansion was observed for all patient donors after the initial day of seeding in normal maintenance media, as well as after subsequent culture in osteogenic media at, respectively, 3, 7, 14 and 28 days in culture (Fig. 1b). AMSCs from all three patients adhered to ps-Ti discs after 1 day, survived for at least 28-days, and migrated into interstitial spaces (Figs. 1c and 2). After 7 days, cells ceased proliferation and maximize ECM production (see below). In addition, cells at the beginning and end of experimental time points illustrate heterogeneity among patient cell donors.

We note that phenotypic variability in cell-cell or cell/ps-Ti contacts among cell populations was apparent after 7 days (Fig. 1b). Our SEM images exhibit variation in the extent of surface coverage. These differences are possibly related to cell number (i.e., at the end of the culturing period), random sampling within each cell population, and/or patient-to-patient heterogeneity. Irrespective of the differences observed by SEM, the consistent adherence and survival of AMSCs on the ps-Ti substrate suggests that they generate a potentially beneficial biological micro-texture (e.g., by expressing ECM proteins; see below).

ps-Ti supports robust cell proliferation and prolonged ECM mRNA production

In addition to nutrients, gases and temperature, the number of cells supported by a culture substrate is directly proportional to the surface area available for colonization. Compared to normal tc-PS, ps-Ti is a three-dimensional substrate that permits expansion in the z-direction, and thus has an increased surface area. Consistent with this increased surface area, greater average RNA yields are evident for cells cultured in osteogenic media on ps-Ti compared to tc-PS. The increased RNA yields in osteogenic medium suggest that the cells are actively proliferating and/or generate more RNA per cell. Cells were not counted directly due to the technical difficulties of reliably extracting all cells that have invaded the interconnected pores of the porous Titanium material. While RNA yields increase on tc-PS until cells begin to reach confluence around day 3, RNA yields continue to rise until a maximum on day 14 for cells grown on ps-Ti discs, as cells colonize the additional surfaces provided by the three-dimensional architecture (Fig. 3a). The 3D surface area of the ps-Ti disc is higher due to the increased surface roughness and rugosity of the materials, although differences in RNA yield may also be caused by increased cell adhesion, proliferative potential and/or metabolic rate of the cells. Examination of RNA integrity by spectrophotometry revealed that RNA remains intact at all days during the 28-day culturing period, indicating that RNA samples at all time-points are primarily derived from living (or recently living) cells.

We tested whether cultured cells can assume one of its two fundamentally distinct biological cellular states (an actively proliferating self-renewing state or confluent metabolically active differentiated state). As a biomarker of cell cycle progression and proxy for proliferation, we measured expression levels of Cyclin B2 (*CCNB2*) throughout the 28-day time course for

both ps-Ti and tc-PS samples. *CCNB2* reaches its maximum expression on day 3 in cells grown on ps-Ti, while cells grown on tc-PS reach this maximum three days earlier at the start of the time course (day 0; Fig. 3b). As a representative mRNA for histone proteins (which compact newly replicating DNA during S phase in mitotically dividing cells), we analyzed a robustly expressed and the cell cycle controlled H4 variant (*HIST2H4A*). The *HIST2H4A* mRNA exhibits a similar pattern of initial down-regulation for cells cultured on tc-PS and an overall increase to a maximum on day 3 for cells cultured on ps-Ti (Fig. 3b).

We have previously shown that actively dividing AMSCs grown on tc-PS express mostly cytoskeletal and chromatin-related proteins, while high level expression of most ECM proteins appears to be restricted to non-dividing confluent cells [13]. Therefore, we anticipated that loss of cell cycle markers, which reflects the cessation of cell division of AMSCs on ps-Ti, would be followed by the production of mRNAs for ECM proteins during osteogenic differentiation. We measured expression of genes involved in ECM production, such as collagenous proteins (e.g., collagen type I $\alpha 1$ (*COL1A1*) and collagen type III $\alpha 1$ (*COL3A1*)). Maximal expression of *COL1A1* and *COL3A1* occur later in cells grown on ps-Ti (days 7-14) than tc-PS (days 3-7) (Fig. 3c), and also decrease more rapidly in cells grown on tc-PS. Taken together, RNA yields and mRNA expression results indicate that AMSCs grown on tc-PS tend to reach a maximum proliferative potential and start producing ECM sooner, but they achieve greater proliferation and sustain ECM production longer when seeded on ps-Ti.

Non-collagenous ECM production is not diminished by ps-Ti

We tested whether osteogenic differentiation of mesenchymal stromal/stem cells on ps-Ti results in expression of non-collagenous ECM proteins. Due to increased RNA yield on ps-Ti discs, we predicted that production of mRNAs for ECM proteins would be prolonged. We measured expression levels of representative non-collagenous biomarkers. For all three patient cell populations grown on ps-Ti, phenotypic changes related to proliferation and ECM synthesis could be detected in patterns similar to those grown on tc-PS (Fig. 4). For example, mRNA expression levels of non-collagenous bone-related extracellular matrix proteins (alkaline phosphatase liver/bone/kidney (*ALPL*), integrin-binding sialoprotein (*IBSP*), matrix gla protein (*MGP*), aggrecan (*ACAN*), and adiponectin (*ADIPOQ*), biglycan (*BGN*), and decorin (*DCN*)) on ps-Ti were not diminished in comparison to tc-PS (Fig. 4). Also, the mRNA levels of each non-collagenous ECM protein followed unique temporal patterns and exhibited intricate differences between cells grown on tc-PS versus ps-Ti (Fig. 4). The main finding of these temporal gene expression profiles for non-collagenous ECM proteins is that production of mRNAs for ECM proteins by cells grown on ps-Ti is robust and follows temporal patterns distinct from cells grown on tc-PS.

Mesenchymal identity is maintained on both culture substrates

Three-dimensional substrates can change the cell surface and physical structure of mesenchymal stromal/stem cells. To verify the mesenchymal identity of AMSC populations grown on ps-Ti and tc-PS surfaces, we assessed gene expression levels of actin, alpha 2, smooth muscle, aorta (*ACTA2*), endosialin/Cluster of Differentiation 248 (*CD248*) and Cluster of Differentiation 44 (*CD44*). Expression levels were similar for all three patients in

both microenvironments (Fig. 5). These results suggest that AMSCs maintain their mesenchymal identity independently of the culture substrate.

TFs targeted by BMP2 are differentially expressed in tc-PS and ps-Ti

Phenotypic differences between cells grown on tc-PS and ps-Ti are evident from changes in gene expression that are controlled by transcription factors. To begin assessing gene regulatory events, we screened a large panel of transcription factors known to regulate the subcellular processes of mesenchymal stromal/stem cells. Within this panel, we found that the distal-less homeobox 3 (*DLX3*), and its paralog distal-less homeobox 5 (*DLX5*), increase over time in cells grown on tc-PS for all three patients, whereas, they decreased in cells grown on ps-Ti. Inhibitor of DNA binding 3 (*ID3*), a dominant negative helix-loop-helix protein, showed the opposite pattern of fold-change for cells cultured on tc-PS versus ps-Ti, consistent with findings for all three patients (Fig. 6). Interestingly, *DLX3*, *DLX5* and *ID3* are all important targets of bone morphogenetic protein 2 (*BMP2*). Thus, genes involved in *BMP2* signaling (e.g., *DLX3*, *DLX5* and *ID3*) appear to exhibit ps-Ti dependent changes in expression. Also of interest, the runt-related transcription factor 2 (*RUNX2*) is genetically required for bone and stimulated during osteoblast differentiation, but this marker is not unique for bone, and broadly expressed in mesenchymal cells. Although *RUNX2* is robustly expressed throughout the time course, its levels do not appear to be modulated (i.e., fold-change indicated in Fig. 6).

Changes in expression of epigenetic regulators on ps-Ti discs

Adaptability of mesenchymal cells to *in vitro* culture substrates may be driven by epigenetic modifications controlled by histone modifying enzymes [13, 14]. To assess whether epigenetic modifications are associated with observed phenotypic differences among AMSCs cultured on tc-PS or ps-Ti, we performed a limited expression screen of epigenetic regulators based on previously published data regarding their important role in bone formation and homeostasis [34, 35]. We monitored mRNA levels for proteins known to influence the osteogenic differentiation of these cells [13] by either depositing transcriptionally repressive chromatin marks through trimethylation of histone H3 lysine 27 (H3K27) or lysine 9 (H3K9). Enhancers of Zeste 1 and Zeste 2 in the Polycomb Repressive Complex 2 Subunit (*EZH1* & *EZH2*), which mediate and maintain cell pluripotency and plasticity through H3K27 methylation [34] show consistent patterns of up and down regulation, respectively, based on fold-change comparisons (Fig. 7). Suppressors of Variegation 3-9 Homolog 1 and Homolog 2 (Drosophila) (*SUV39H1* & *SUV39H2*), which mediate transcriptional gene silencing through H3K9 methylation [35], were less consistently expressed among patients. Our results suggest selective differences in the expression of epigenetic regulators when cells are cultured on ps-Ti and tc-PS.

DISCUSSION

Complications in patients with major bone deficiencies, diabetes, osteopenia, osteoporosis, and/or impaired bone healing (e.g., when smoking or receiving chemotherapy/radiation) often lead to challenging primary and revision surgeries. These patients would indirectly benefit from *in vitro* validation of combining an FDA-approved implant material with

human AMSCs prepared under Good Manufacturing Practice guidelines, if the technology progressed to animal model and human clinical trial. Among metal biomaterials (e.g., highly porous tantalum, trabecular metal), ps-Ti has a favorably high coefficient of friction and surface rugosity, while its material strength mimics the elastic modulus of bone. Therefore, our study was designed to investigate the potential for improving a currently used implant material by seeding AMSCs to ultimately achieve rapid and ubiquitous osseointegration. We uncovered the potential of this technique by revealing that human AMSCs can be successfully seeded and expanded on the metal, while preserving their physicochemical identity, and importantly, their osteogenic potential.

AMSCs attach to ps-Ti

To assess strategies for enhancing orthopedic implant osseointegration, we examined whether AMSCs are capable of adhering to ps-Ti surfaces. We found that these cells clearly have potential use as a coating material for orthopedic bone implants. We selected clinical-grade AMSCs grown in human platelet lysate because these cells are in ample supply, are capable of tri-lineage differentiation [12], and have trophic actions that may aid in recruiting other cells that regenerate bone [8]. The timing of lateral surface coverage expansions on the ps-Ti substrate varied depending on patient donor, yet ps-Ti supports three-dimensional substrate integration. Also on ps-Ti, AMSCs exhibited delayed cessation of proliferation, prolonged expression of mRNAs related to ECM production, and retained mesenchymal identity, suggesting therapeutic relevance *in vivo*.

When metallic implants interact with cells through focal adhesions, this contact should trigger integrin-dependent activation of kinase pathways. These kinases ultimately communicate with gene regulatory pathways by phosphorylating transcription factors and/or epigenetic regulators that function as molecular endpoint effectors in the nucleus. Stable production of known *BMP2* targets (*DLX5* and *ID3*), were observed in cells grown on ps-Ti. *EZH1* and *EZH2* exhibit differences in expression on ps-Ti compared to standard tc-PS, suggesting a potentially important role for the culture substrate to alter epigenetic regulation. Because transcription factors and epigenetic regulators control the osteoblast phenotype and bone-related ECM gene expression by altering molecular memory at the level of chromatin structure, we envision that modulating the activities of these gene regulatory factors by inhibition may permit beneficial manipulations of the osteogenic potential of ps-Ti-seeded AMSCs.

Optimizing cell growth and differentiation on ps-Ti

The rugosity of ps-Ti increases the three-dimensional surface area accessible to cells. Nevertheless, cell coverage of ps-Ti was observed on a simple disc as a model for stem cell growth on an orthopedic ps-Ti implant. Although ps-Ti exhibits considerable structural complexity by SEM, we show that AMSCs from three different patients are capable of adhering to ps-Ti and continuously cover the surface within two to three weeks. We also show that when AMSCs differentiate on ps-Ti, they produce high levels of mRNAs for bone-related collagenous and non-collagenous proteins (e.g., collagens and proteoglycans), as well as paracrine signaling ligands (e.g., cytokines and chemokines). Recent data from our group indicate that these bone-anabolic functions of AMSCs can be enhanced by inhibiting

epigenetic enzymes [36]. Thus, it is feasible to design strategies to test the role of specific epigenetic inhibitors on expression of bone-related ECM proteins during osteogenic differentiation of AMSCs on ps-Ti in three-dimensional culture.

Trophic paracrine activities of AMSCs

AMSCs are trophically active, yet the specific trophic functions of cells attached to biomaterials are not well documented. Specifically, cells should alter the implant surface microenvironment by coating the ps-Ti surface with biosynthetically produced ECM proteins, and by secreting paracrine factors that will either recruit other mesenchymal progenitors or inactivate inflammatory cells [8]. Experimentally validating these scenarios would be a logical next step toward building on the results presented here. Likewise, some cells and surface coatings have an exogenous antibacterial action when attached to biomaterials. In the case of ps-Ti, this hypothesis is testable by co-culture experiments designed to measure the “race for the surface” by AMSCs in comparison to specific pathogenic bacteria [37].

Additional metallic biomaterials may positively interact with AMSCs to improve osseointegration (e.g., tantalum, cobalt chromium, nitinol, plastic polymers, and hydrogels), or materials with tethered paracrine factors (or other agents) to improve the microenvironment. By modulating culture conditions (e.g., oxygen tension), or using other mesenchymal cell types or sources it may be possible to further optimize cell culture conditions. Nevertheless, the results presented here provide the *in vitro* validation necessary for further *in vitro* experimentation and functional osseointegration modeling *in vivo*.

Conclusion

Innovative improvements in implant technologies are necessary to address surgical complications resulting from more than 4 million total hip and knee replacements expected by 2030 in the US [4]. Revision joint surgeries are sometimes necessary and associated with major bone loss and/or infection, thus reducing options for fixation of revision components. Responding to this pressing clinical need, we used clinical-grade mesenchymal stromal/stem cells to effectively coat a surgical-grade ps-Ti implant material. By concluding that ps-Ti implants can be biologically enhanced using AMSCs pre-committed to express the osteoblastic phenotype; we provide the preliminary information necessary for preclinical animal modeling, and by extension, preclinical FDA-approval for progression toward a human trial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

AMSCs	adipose-tissue derived mesenchymal stromal/stem cells
ECM	extracellular matrix
tc-PS	tissue culture polystyrene
ps-Ti	porous structured titanium
PBS	phosphate buffered saline
RT-qPCR	Quantitative real-time PCR
SEM	Scanning electron microscopy
CCNB2	Cyclin B2
HIST2H4A	Histone Cluster 2, H4a
COL1A1	collagen type I α 1
COL3A1	collagen type III α 1
ALPL	alkaline phosphatase liver/bone/kidney
IBSP	integrin-binding sialoprotein
MGP	matrix gla protein
ACAN	aggrecan
ADIPOQ	adiponectin
BGN	biglycan
DCN	decorin
ACTA2	actin, alpha 2, smooth muscle, aorta
CD248	endosialin/Cluster of Differentiation 248
CD44	Cluster of Differentiation 44
DLX3	distal-less homeobox 3

DLX5	distal-less homeobox 5
ID3	Inhibitor of DNA binding 3
BMP2	bone morphogenetic protein 2
RUNX2	Runt-Related Transcription Factor 2
H3K27	histone H3 lysine 27
H3K9	histone H3 lysine 9
EZH1	Enhancers of Zeste 1 in the Polycomb Repressive Complex 2 Subunit
EZH2	Enhancers of Zeste 2 in the Polycomb Repressive Complex 2 Subunit
SUV39H1	Suppressor of Variegation 3-9 Homolog 1 (Drosophila)
SUV39H2	Suppressor of Variegation 3-9 Homolog 2 (Drosophila)

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HIGHLIGHTS

1. We modeled growth and differentiation of adipose-derived mesenchymal stromal cells (AMSCs) on orthopedic metallic implants
2. Electron microscopy and histology reveal that AMSCs adhere to porous titanium
3. AMSCs cultured on titanium exhibit altered cell cycle progression
4. Extracellular matrix production is enhanced on the titanium microenvironment
5. AMSCs maintain phenotypic identity as indicated by expression of key osteoblastic transcription factors and epigenetic regulators.

Figure 1.

(a) Scanning electron microscopy images of porous structured titanium (TiAl6V4) at 1x, 30x, 200x, and 1500x. (b) SEM images after 7 days in culture at 30x, 200x, and 1300x. (c) Histology slide of Sanderson's rapid bone stain demonstrating vertical penetration into pores of ps-Ti to an approximate depth of 1 mm. Samples were taken from donor AMSC-258 after 14 days. Close-up image of Sanderson's Rapid Bone Stain (Collagen = Grey).

Figure 2. Scanning electron microscopy images (30x resolution) of highly porous structured titanium (TiAl6V4) seeded with adipose tissue derived mesenchymal stromal/stem cells (AMSCs) from three different human donors: after 1 day in standard maintenance medium (left), or 4 weeks in osteogenic medium (right).

Figure 3.

(a) Average RNA Yield (ng/mm²) on porous structured Ti (ps-Ti) vs tissue culture polystyrene (tc-PS) for a representative donor (AMSC 283). RNA yields were standardized relative to the respective 2D surface areas of standard 6-well tissue culture plate (951 mm²) and ps-Ti discs (491 mm²). mRNA yields increased considerably by day 14 on ps-Ti compared to tc-PS samples. (b) Cell cycle markers (*CCNB2*, *HIST2H4*). (c) ECM production by collagenous bone-related proteins (*COL1A1*, *COL3A1*).

Figure 4.
Production of genes related to ECM. Non-collagenous bone-related proteins: *ALPL*, *IBSP*, *MGP*, *ACAN*, *ADIPOQ*, and small leucine-rich proteoglycans: *BGN*, and *DCN*.

Figure 5.
Identity of AMSCs based on mesenchymal lineage markers: *ACTA2*, *CD248*, and *CD44*.

Figure 6. Screen of gene expression for 20 transcription factors (fold change of expression on D21 vs D0). Time course analysis of mRNA levels for *DLX3*, *DLX5*, and *ID3* using AMSC 211.

Figure 7. Mini-screen of epigenetic regulators related to bone (*EZH1*, *EZH2*, *SUV39H1*, and *SUV39H2*) as fold change of expression on D21 vs D0. Time course data showing temporal changes in mRNA levels consistent with results of the analysis of selected epigenetic regulators on tc-PS versus ps-Ti for a representative patient (AMSC 211).

Table 1

An overview of donor data and sampling for gene expression studies

ID	211		237		283	
Age	41		60		54	
Gender	M		F		M	
Disease	None		None		None	
Harvest Site	Abdomen		Abdomen		Abdomen	
Culture Treatment	<u>tc-PS</u>	<u>ps-Ti</u>	<u>tc-PS</u>	<u>ps-Ti</u>	<u>tc-PS</u>	<u>ps-Ti</u>
Day -1 (seeding/subconfluent)	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥
Day 0 (osteo. cocktail)	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥
Day 3	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥
Day 7	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥
Day 14	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥
Day 21	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥
Day 28	♣	♣ ♥	♣	♣ ♥	♣	♣ ♥

(♣ = RT-qPCR data; ♥ = SEM; tc-PS = tissue culture polystyrene; ps-Ti = porous structured titanium).

Table 2

Sequences for primers used in qPCR reactions

Gene ID	Class / Biology	Forward Primer	Reverse Primer
<i>ACAN</i>	B / Cartilage & ECM	GTGCCTATCAGGACAAGGTCT	GATGCCTTTCACCACGACTTC
<i>ACTA2</i>	B / Smooth muscle	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC
<i>ADIPOQ</i>	B / Fat	AACATGCCCATTGCTTTACC	TAGGCAAAGTAGTACAGCCCA
<i>ALPL</i>	B / Bone	ACTGGTACTCAGACAACGAGAT	ACGTCAATGTCCCTGATGTTATG
<i>ATF1</i>	TF	CTGGAGTTTCTGCTGCTGTC	GGCAATGGCAATGTACTGTC
<i>ATF4</i>	TF	ATGACCGAAATGAGCTTCCTG	CTGGAGAACCCATGAGGTTTG
<i>BGN</i>	B / ECM	GAGACCCTGAATGAACTCCACC	CTCCCGTTCTCGATCATCCTG
<i>CCNB2</i>	B / Cell cycle	CCGACGGTGTCCAGTGATTT	TGTTGTTTTGGTGGGTGAACT
<i>CD248</i>	B / Cell surface	ATCGCAGCCAACTATCCAGAT	TTCCAGGCAAATGAGTGGTGG
<i>CD44</i>	B / Cell surface	CTGCCGCTTTGCAGGTGTA	CATTGTGGGCAAGGTGCTATT
<i>COL1A1</i>	B / Bone	GTAACAGCGGTGAACCTGG	CCTCGCTTTCCTTCTCTCC
<i>COL3A1</i>	B / Bone	TTGAAGGAGGATGTTCCCATCT	ACAGACACATATTTGGCATGGTT
<i>DCN</i>	B / Cartilage	ATGAAGGCCACTATCATCCTCC	GTCGCGGTCATCAGGAACTT
<i>DLX3</i>	TF	TACCCTGCCGAGTCTTCTG	GCCCGAGTAGTAATCGTGCTG
<i>DLX5</i>	TF	AGCTACGCTAGCTCCCTACCACC	GGTTTGCCATTCAACATTCTCAC
<i>EZH1</i>	ER / Me writer	GACATGCTATTGAAGGCAACA	GACACCATTCTCAGGGAACAT
<i>EZH2</i>	ER / Me writer	GACGGCTTCCCAATAACAGTA	AGTCCCTGCTTCCCTATCACT
<i>FOSL1</i>	TF	GCCGCCCTGTACCTTGTATCT	CAGTGCCTCAGGTTCAAGCA
<i>GAPDH</i>	Norm / Housekeeping	ATGTTCTGTCATGGGTGTGAA	TGTGGTCATGAGTCCTTCCA
<i>HIST2H4A</i>	B / Cell cycle	AGCTGTCTATCGGGCTCCAG	CCTTTGCCTAAGCCTTTTCC
<i>IBSP</i>	B / Bone	GAACCTCGTGGGGACAATTAC	CATCATAGCCATCGTAGCCTTG
<i>ID1</i>	TF	CTGCTCTACGACATGAACGG	GAAGGTCCCTGATGTAGTCGAT
<i>ID3</i>	TF	GAGAGGCACTCAGCTTAGCC	TCCTTTTGTGCTTGGAGATGAC
<i>KLF10</i>	TF	CTTCCGGGAACACCTGATTTT	GCAATGTGAGGTTTGGCAGTATC
<i>MEF2C</i>	TF	TCGTGGAGACGTTGAGAAAG	TTCTTGTTCATGCGGAATC
<i>MGP</i>	B / ECM	TCCGAGAACGCTCTAAGCCT	GCAAAGTCTGTAGTCATCACAGG
<i>MSX2</i>	TF	ATGGCTTCTCCGTCCAAAGG	TCGTGCGGCGAAAACAAGTC
<i>NKX3-1</i>	TF	CCCACACTCAGGTGATCGAG	GAGCTGCTTTCGCTTAGTCTT
<i>PRRX1</i>	TF / MSC phenotype	CAGGCGGATGAGAACGTGG	AAAAGCATCAGGATAGTGTGTCC
<i>RUNX1</i>	TF	CTGCCCATCGCTTTCAAGGT	GCCGAGTAGTTTTTCATCATTGCC
<i>RUNX2</i>	TF	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
<i>RUNX3</i>	TF	AGGCAATGACGAGAACTACTCC	CGAAGGTCGTTGAACCTGG
<i>SATB2</i>	TF	GTGCCCCGATGGTTACACC	CTTCTACAAATATGGCGGCAT
<i>SOX4</i>	TF	AGCGACAAGATCCCTTTCATTC	CGGGGTAGTCAGCCATGTG
<i>SOX9</i>	TF	TGTATCACTGAGTCATTTGCAGTGT	AAGGTCTGTGAGTGGGCTGAT
<i>SP1</i>	TF	CAGGTGCAAACCAACAGATTA	GCTGGAGTAGGTTTGGCATAG
<i>SUV39H1</i>	ER / Me writer & reader	CCTGCCCTCGGTATCTCTAAG	ATATCCACGCCATTTACCAG
<i>SUV39H2</i>	ER / Me writer & reader	TCTATGACAACAAGGGAATCACG	GAGACACATTGCCGTATCGAG
<i>TRPS1</i>	TF / MSC phenotype	ATGACACTCCTGTTGGGTACT	CGTGCTGCTTGCCATAATGTT

(B = biomarker; TF = transcription factor; ER = epigenetic regulator; Me = methylation; MSC = mesenchymal stromal/stem cell).

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