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Mediation of Osteogenic Differentiation of Human Mesenchymal Stem Cells on Titanium Surfaces by a Wnt-Integrin Feedback Loop

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Abstract

Peri-implant bone formation depends on the ability of mesenchymal cells to colonize the implant surface and differentiate into osteoblasts. Human mesenchymal stem cells (HMSCs) undergo osteoblastic differentiation on microstructured titanium (Ti) surfaces in the absence of exogenous factors, but the mechanisms are unknown. Wnt proteins are associated with an osteoblast phenotype, but how Wnt signaling regulates HMSC differentiation on microstructured Ti surfaces is not known. HMSCs were cultured on tissue culture polystyrene or Ti (PT [Sa=0.33μm, θ=96°], SLA [Sa=2.5μm, θ=132°], modSLA [hydrophilic-SLA]). Expression of calcium-dependent Wnt ligand WNT5A increased and canonical Wnt pathway ligands decreased on microstructured Ti in a time-dependent manner. Treatment of HMSCs with canonical ligand Wnt3a preserved the mesenchymal phenotype on smooth surfaces. Treatment with Wnt5a increased osteoblastic differentiation. Expression of integrins ITGA1, ITGA2, and ITGAV increased over time and correlated with increased WNT5A expression. Treatment of HMSCs with Wnt5a, but not Wnt3a, increased integrin expression. Regulation of integrin expression due to surface roughness and energy was ablated in WNT5A-knockdown HMSCs. This indicates that surface properties regulate stem cell fate and induce osteoblast differentiation via the Wnt calcium-dependent pathway. Wnt5a enhances osteogenesis through a positive feedback with integrins and local factor regulation, particularly through BMP signaling.

Keywords

Cell signaling; Surface roughness; Titanium; Stem cell; Growth factors

INTRODUCTION

The first event after a biomaterial implantation is the interaction between the surface and proteins, carbohydrates, lipids, and ions from the blood and serum. These interactions are

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dictated by the chemical composition, surface energy, and micro-nanotopography of the implant surface [1–3]. Studies using a variety of osteoblast cell culture models have shown that these cells exhibit a more differentiated phenotype when grown on titanium substrates in comparison to tissue culture polystyrene, reflecting differences in surface properties [4, 5]. The osteoblasts do not come into direct contact with the biomaterial but instead interact with the proteins adsorbed to the surface via integrin signaling [6, 7] and studies suggest that integrin signaling is responsible for surface-dependent osteoblastic maturation. Knock down of the beta1 ($\beta 1$) or alpha2 ($\alpha 2$) integrin subunits in osteoblasts impairs the ability of osteoblasts to recognize complex microstructured titanium (Ti) surfaces, causing the cells to fail to differentiate or produce an osteogenic environment [8, 9].

These observations suggest that the composition of the adsorbed protein layer can also affect progenitor cell lineage fate [10]. Among the first cells to colonize the implant surface are multipotent progenitor cells that migrate through the peri-implant clot [11, 12]. Previously we showed that human mesenchymal stem cells (HMSCs) undergo osteoblastic differentiation in response to Ti surface microstructure and energy, even in the absence of exogenous factors typically used to induce osteogenesis [13]. One possible mechanism regulating this process is altered gene transcription due to integrin-dependent changes in cytoskeleton, altering downstream cell responses [14]. We found that signaling via $\alpha 2\beta 1$ is required for osteoblasts cultured on microstructured Ti to produce soluble paracrine/autocrine factors involved in osteoblast differentiation of HMSCs [15–17]. Moreover, Wnt pathway inhibitors Dkk1 and Dkk2 modulate osteoblastic maturation on microstructured titanium surfaces, and the effect is dependent on cell maturation state [18]. Surprisingly, Dkk2 but not Dkk1 was required for osteoblast differentiation on the microtextured Ti substrates. Similarly, Dkk2 produced by osteoblasts cultured on microtextured Ti was required for osteoblastic differentiation of HMSCs cultured on TCPS [18].

This observation suggested that osteoblastic differentiation of HMSCs on microstructured Ti and in the absence of osteogenic supplements like beta-glycerophosphate or dexamethasone might involve a mechanism different from that required for osteoblastic differentiation on TCPS, where osteogenic supplements are routinely used. To address this hypothesis, we focused on the role of Wnt proteins. The Wnt family of proteins regulates patterning, development, proliferation, and differentiation in a large variety of organs and tissues [19]. These proteins have both autocrine and paracrine functions, binding to G-protein coupled frizzled family (Fzd) receptors, which triggers downstream pathways according to the specific ligand-receptor combination. Wnt protein effects are both by the ligand as well as the specific receptor-co-receptor interaction. Fzd receptors couple with the co-receptors low-density lipoprotein receptor related proteins (LRP5/6), receptor tyrosine kinase-like orphan receptor 2 (Ror2), and receptor-like tyrosine kinase (Ryk) to initiate signaling.

Wnt signaling is transduced through one of three pathways: the canonical β -catenin pathway, the calcium-dependent pathway, or the planar cell polarity pathway. In the current study, we focused on the canonical and calcium dependent pathways. Canonical Wnt signaling is transduced when a Wnt protein binds a Fzd receptor and a co-receptor (Lrp5 or Lrp6), activating Dishevelled (Dsh) and inhibiting the Axin/GSK3 β /APC complex. While GSK3 β normally phosphorylates β -catenin, targeting it for degradation, activation of Dsh blocks this. This leads to β -catenin accumulation in the cytoplasm and subsequent translocation into the nucleus, where it modulates gene transcription. This pathway has been shown to be important in osteogenic differentiation of mesenchymal stem cells and bone formation/development [20–23].

In calcium-dependent Wnt signaling, Wnts stimulate intracellular Ca^{2+} release, activating calmodulin-dependent kinase II (CamKII), protein kinase C (PKC), and calcineurin. This

initiates a signaling cascade that modulates gene expression. Wnt5a^{+/-} mice have reduced bone mass, fewer osteoblasts, and more adipocytes than wild type mice [24]. These observations suggest that Wnt5a blocks PPAR- γ -induced adipogenesis in bone marrow cells, thus inducing osteoblastogenesis, and that it increases the commitment of mesenchymal stem cells to the osteoblast phenotype [24–26].

In this study, we examined the time course of expression of Wnt pathway molecules in HMSCs and the potential contributions of Wnt3a and Wnt5a (Wnt canonical and non-canonical pathway ligands, respectively) in their differentiation on microstructured titanium surfaces. Furthermore, we evaluated the autocrine and paracrine effects of Wnt3a and Wnt5a by blocking the intrinsic proteins with specific antibodies. Finally, we explored the interaction between integrins and Wnt signaling in osteogenic differentiation of HMSCs cultured on microstructured Ti.

MATERIALS AND METHODS

Ti Disk Preparation

Ti disks 15mm in diameter were punched from 1mm thick sheets of grade 2 unalloyed titanium and supplied to us by Institut Straumann AG (Basel, Switzerland). Disks were prepared as described previously and sterilized overnight with 25-kGy gamma irradiation [27]. Briefly, disks were degreased in acetone and processed for 30 s in a 55°C 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution to produce pretreatment Ti disks (PT). PT disks were grit-blasted with 0.25–0.50 mm corundum grit at 5 bar and acid etched in HCl/H₂SO₄ to prepare SLA substrates. modSLA substrates were prepared in the same manner, but were rinsed under nitrogen and stored in an isotonic saline solution

Surface Characterization

PT and SLA surfaces were characterized as received from Institut Straumann. Since modSLA surfaces are stored in isotonic saline, disks were rinsed for three minutes three times with ultrapure H₂O in an ultrasonic bath and then dried with N₂.

Surface morphology of Ti disks was examined by scanning electron microscopy (SEM) using an Ultra 60 field emission (FE) microscope (Carl Zeiss SMT Ltd., Cambridge, UK). Images were recorded using a 5 kV accelerating voltage. X-ray photoelectron spectroscopy (XPS) measurements were performed on a Thermo K-Alpha (Thermo Fisher Scientific Inc., MA). The XPS analysis chamber was evacuated to a pressure of 10⁻⁹ Torr or lower before collecting XPS spectra. This system was equipped with a monochromatic Al K α X-ray source ($h\nu = 1486.6$ eV photons) at a 90° takeoff angle. XPS results were evaluated using the Thermo Advantage 4.43 software package provided by Thermo Fisher Scientific, Inc.

Contact angles of Ti disks were determined by Ramé-Hart goniometer (model 250-F1, NJ, USA). Images were analyzed with DROPimage CA software package (Ramé-Hart Instrument Co., Netcong, NJ).

Surface roughness of Ti disks was measured using a LEXT 3D Material Confocal Microscope (Olympus America Inc., PA). A 100X objective was used and the field of view was 128 $\mu\text{m} \times 128 \mu\text{m}$. Roughness results were evaluated using the LEXT OLS4000 software (Olympus). Skewness (Ssk), a measure of the symmetry of peaks and valleys [28, 29] was also measured. Three measurements were made on each of two surfaces per group.

Cell Culture Methods

Bone marrow HMSCs (Lonza, Walkersville, MD) were plated at 5,000 cells/cm² on TCPS, PT, SLA, or modSLA surfaces and cultured in Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza). Cells were cultured at 37°C with 5% CO₂ and 100% humidity.

Wnt5a Silencing

HMSCs were transduced with shRNA lentiviral transduction particles (SHCLNV-NM_003392, Mission®, Sigma Aldrich, St. Louis, MO) to silence WNT5A. HMSCs were plated at 20,000 cells/cm² and cultured overnight as above. Particles were added to the cells at a multiplicity of infection of 7.5. After an 18 hour incubation, transduced cells were selected with 0.25 µg/ml of puromycin. Silencing of WNT5A was confirmed using real-time qPCR as described below.

Autocrine and Paracrine Effects of Wnt3a and Wnt5a

To test the effect of exogenous Wnts on our model system, we treated cultures with either 50 ng/mL recombinant human Wnt3a (R&D Systems, Minneapolis, MN) or 125 ng/mL recombinant mouse Wnt5a (R&D Systems). To test the effect of endogenous Wnts, we treated with 1:200 dilutions of either a rabbit polyclonal antibody to Wnt3a [AbWnt3a] (Abcam ab28472, Cambridge, MA) or a rabbit polyclonal antibody to Wnt5a [AbWnt5a] (Abcam ab72583). Media were changed every 48 hours and cells treated with either proteins or antibodies to Wnts until they reached confluence on TCPS, about 7 days. At confluence, cells were incubated in full media without treatment for 24h. The conditioned media were collected and osteocalcin levels measured by radioimmunoassay (Biomedical Technologies Inc., Stoughton, MA). Levels of OPG (R&D Systems), VEGF (R&D Systems), TGF-β1 (R&D Systems), BMP2 (PeproTech, Rocky Hill, NJ) and BMP4 (R&D Systems) in the conditioned media were measured by ELISA per manufacturer's instructions, as described previously [30, 31]. Cells were harvested from the surfaces by two sequential trypsinizations and counted. Cells were lysed and alkaline phosphatase specific activity and protein levels measured in the lysate. Both cell types were treated with 1:200 dilutions of IgG; results were not significantly different from untreated cells (data not shown).

Wnt Pathway Gene Expression

To determine whether Wnt pathway genes were regulated during differentiation of HMSCs on microstructured Ti substrates, we performed three studies. In the first, HMSCs were plated on TCPS or Ti disks and cultured until cells reached confluence on TCPS. Cells were incubated with fresh media for 12 hours and harvested using TRIzol (Invitrogen). In a second study, a time-course of expression was determined. HMSCs were plated on TCPS or Ti disks and were harvested after two, four, or six days of culture. Cells were incubated with fresh media for 12 hours on day of harvest and RNA extracted using the RNAqueous®-Micro RNA extraction kit (Applied Biosystems, Carlsbad, CA). Finally, the effect of Wnt3a and Wnt5a on HMSC gene expression was determined. HMSCs were plated on TCPS. At confluence, cells were treated with 50 ng/ml rhWnt3a or 50 ng/ml rhWnt5a for 12 hours. RNA was isolated using TRIzol.

For all studies, RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). To create a cDNA template, 125 ng of RNA was reverse transcribed using a High Capacity Reverse Transcription cDNA kit (Applied Biosystems). To quantify expression of Wnt ligands, receptors, co-receptors, and inhibitors, cDNA was used for real-time PCR with gene-specific primers using the StepOnePlus Real-time PCR System and *PowerSybr®* Green Master Mix (Applied Biosystems). Fluorescence values were quantified as starting quantities using known dilutions of HMSCs grown on TCPS.

Genes are presented as normalized to GAPDH. Primers (Supplemental Table 1) were designed using the Beacon designer software and synthesized by Eurofins MWG Operon (Huntsville, AL).

RESULTS

Surface Characterization

SEM and confocal microscopy demonstrate that SLA and modSLA do not have significantly different morphological and topographical features. However, the surface chemical composition of the modSLA surfaces, altered through manufacturing, created surfaces with different surface wettability and energy but identical topography.

SEM images of SLA and modSLA surfaces showed complex micron (10–50 μm) and submicron (1–2 μm) scale structures (Fig. 1A). However, on high magnification (lower panel), small smooth areas were found on both surfaces. PT surfaces were relatively smooth and planar in comparison to SLA and modSLA substrates (Fig. 1A, left panel). SEM images confirmed morphological differences between PT and SLA surfaces, but no difference between SLA and modSLA disks.

XPS survey spectra indicated titanium (Ti), oxygen (O), and carbon (C) as the main components of all surfaces. Ti surfaces have very thin oxide layer consisting mainly of TiO_2 [32]. The chemical composition (Fig. 1B) varied between PT (left panel), SLA (center panel), and modSLA (right panel) surfaces. On modSLA surfaces, carbon contamination decreased and oxygen concentration increased compared to PT or SLA surfaces. High resolution analysis of C(1s) spectra of modSLA surface indicated aliphatic carbons due to adsorption of carbon-containing molecules from the air [32]. Contact angles were $96^\circ \pm 1.6$ for PT, $132^\circ \pm 2.5$ for SLA, and 0° for modSLA, indicating that the PT and SLA surfaces are hydrophobic while the modSLA substrate is super-hydrophilic, confirming previous findings [4].

Three-dimensional confocal microscopy confirmed that the PT surface has a different morphology than the SLA and modSLA surfaces (Fig. 1D; surface negative: upper panel; surface positive: lower panel). The roughness of PT substrates ($S_a = 0.33 \mu\text{m}$) was significantly lower than SLA ($S_a = 2.5 \mu\text{m}$). However, there was no difference between SLA ($S_a = 2.5 \mu\text{m}$) and modSLA ($S_a = 2.7 \mu\text{m}$). SLA and modSLA surfaces had high peaks and low valleys, features not found on PT substrates. Symmetrical distribution of peaks and valleys seen on SLA ($S_{sk} = 0.09$) and modSLA ($S_{sk} = 0.09$) was not present on PT surfaces ($S_{sk} = -0.48$), which instead featured deep scratches on the surface.

Effect of Surface Topography and Hydrophilicity on Wnt Pathway Gene Expression

At confluence, expression of several Wnt receptors, ligands, and downstream molecules were measured. Expression of the canonical Wnt ligands WNT1, WNT3A, and WNT7B were downregulated on the rough SLA and modSLA surfaces in comparison to TCPS and PT (Fig. 2A, 2B, 2D), as was the downstream canonical molecule CTNNB (Fig. 2E). In contrast, expression of the non-canonical, calcium-dependent Wnt pathway ligand WNT5A was upregulated on rough surfaces in comparison to smooth TCPS or PT (Fig. 2C). AXIN2, a negative regulator of the canonical Wnt signaling pathway, was also upregulated on rough surfaces in comparison to TCPS or PT (Fig. 2F), indicating suppression of canonical Wnt signaling and activation of the calcium-dependent Wnt signaling pathway in HMSC osteoblastic differentiation on microstructured Ti surfaces.

Expression of FZD1, FZD3, and FZD4 was not modulated in cells on Ti substrates (Fig. 2G, 2I, 2J). FZD2 expression increased in cells on SLA and modSLA surfaces in comparison to

TCPS, but was not significantly different from expression on PT (Fig. 2H). FZD5, FZD6, FZD7, and FZD8 increased expression in a roughness-dependent manner, showing higher levels on SLA and modSLA in comparison to TCPS or PT surfaces (Fig. 2K, 2L, 2M, 2N). In contrast, expression of FZD9 was downregulated on Ti substrates (Fig. 2O).

Effect of Wnt3a on HMSC Differentiation

Culture on rough microstructured titanium surfaces decreased HMSC cell number (Fig. 3A), but there was no additional decrease due to surface energy. Treatment with Wnt3a increased cell number 25% on the smooth TCPS and PT surfaces when compared to control, but there was no effect on the cells grown on SLA or modSLA surfaces. AbWnt3a did not affect cell number on any of the surfaces examined. Alkaline phosphatase specific activity increased on SLA and modSLA on control surfaces (Fig. 3B). Addition of Wnt3a increased alkaline phosphatase specific activity when compared to control, with an increase on the rough SLA surface and a synergistic effect on the hydrophilic modSLA surface. Alkaline phosphatase specific activity was not significantly different after addition of AbWnt3a when compared to control. Levels of osteocalcin in the conditioned media were 1.5 fold higher on SLA and modSLA surfaces than on TCPS or PT surfaces (Fig. 3C). Exogenous Wnt3a decreased osteocalcin on all surfaces in comparison to control, but the effect was not dependent on surface topography. Osteocalcin levels of untreated and AbWnt3a treated cells were comparable on TCPS and PT, but increased after AbWnt3a treatment on SLA and modSLA surfaces. OPG levels in untreated cells increased 50% on SLA and modSLA surfaces in comparison to TCPS and PT surfaces (Fig. 3D). Treatment with AbWnt3a did not change the levels from the untreated cells. Wnt3a decreased levels of OPG on all surfaces.

Increased surface roughness caused a 3-fold increase in levels of BMP2 in the conditioned media of cells grown on SLA and modSLA surfaces when compared to TCPS and PT, but levels were the same after treatment with Wnt3a or AbWnt3a when compared to untreated HMSCs (Fig. 3E). BMP4 also exhibited increased levels on rough surfaces, independent of treatment (Fig. 3F). VEGF increased in HMSCs on the titanium PT surface with a further 50% increase on the rough SLA and modSLA surfaces (Fig. 3G). While Wnt3a-treated cells had similar levels of VEGF on TCPS and PT as untreated cells, levels were modestly higher on SLA and modSLA after treatment. Cells treated with AbWnt3a produced lower levels of VEGF on PT, SLA, and modSLA surfaces than untreated cells. Levels of latent TGF- β 1 were independent of treatment on TCPS (Fig. 3H). TGF- β 1 levels were higher in untreated cells on PT surfaces than on TCPS and were additionally increased by the rough titanium SLA and modSLA surfaces. Addition of Wnt3a increased latent TGF- β 1 20% on PT, SLA, and modSLA surfaces as compared to the untreated HMSCs. Treatment with AbWnt3a had no effect on latent TGF- β 1.

Effect of Wnt5a on HMSC Differentiation

Cell number was lower in cultures of HMSCs grown on rough titanium SLA and modSLA surfaces (Fig. 4A). Treatment with Wnt5a increased cell number on TCPS, but were similar to control on PT, SLA and modSLA surfaces. Blocking endogenous Wnt5a with AbWnt5a treatment increased cell number on PT, SLA, and modSLA surfaces as compared to untreated cells. Untreated HMSCs increased alkaline phosphatase specific activity on SLA and modSLA in comparison to TCPS and PT surfaces (Fig. 4B). Exogenous Wnt5a increased alkaline phosphatase specific activity 50% over control on TCPS and PT, but caused a 100% increase on SLA and modSLA surfaces. AbWnt5a-treated cells significantly decreased alkaline phosphatase on SLA and modSLA substrates in comparison to untreated cells, but there was no effect of the treatment on TCPS or PT surfaces. Osteocalcin levels showed a roughness-dependent increase in HMSCs (Fig. 4C). Wnt5a treatment slightly increased osteocalcin on PT, but caused a 33% increase in SLA and modSLA surfaces.

Treatment with AbWnt5a decreased osteocalcin levels only on modSLA surfaces; levels were not statistically different from control on TCPS, PT, or SLA surfaces. OPG levels increased in the untreated cells on rough titanium surfaces (Fig. 4D). Cells treated with Wnt5a did not change levels of OPG on any surface as compared to untreated cells. AbWnt5a increased OPG on TCPS and PT surfaces, but did not affect OPG production in cells on SLA or modSLA surfaces.

BMP2 increased 2-fold in conditioned media of HMSCs grown on SLA and modSLA surfaces as compared to TCPS or PT surfaces (Fig. 4E). Wnt5a increased BMP2 levels on PT, SLA, and modSLA in comparison to untreated HMSCs. BMP2 levels in cells treated with AbWnt5a were unchanged from control on TCPS and PT surfaces; however, levels were decreased 25% on SLA and modSLA surfaces. BMP4 levels increased in HMSCs grown on rough SLA surfaces and were additionally increased on modSLA surfaces (Fig. 4F). Wnt5a-treated cells showed increased BMP4 levels on PT, SLA, and modSLA surfaces, but were unchanged on TCPS, as compared to untreated cells. AbWnt5a-treated cells produced similar levels of BMP4 as the control cells. VEGF increased in untreated cells grown on titanium surfaces, increasing 100% on SLA and modSLA as compared to TCPS (Fig. 4G). There was no change in amounts of VEGF produced when cells were treated with either Wnt5a or AbWnt5a. Latent TGF- β 1 increased on PT, SLA and modSLA surfaces 2-fold over TCPS (Fig. 4H). Although TGF- β 1 levels were similar between Wnt5a-treated and control cells on TCPS, Wnt5a increased TGF- β 1 on PT, SLA, and modSLA surfaces. AbWnt5a did not affect TGF- β 1 levels of cells grown on TCPS, PT, or modSLA substrates, but decreased production in cells grown on SLA.

Time Course of Wnt Pathway Expression on Ti Surfaces

We next wanted to determine whether Wnt signaling regulation occurred during normal differentiation of HMSCs on microstructured Ti substrates. HMSCs were harvested after two, four, or six days of culture and expression of Wnt ligands, receptors, co-receptors, and inhibitors as well as two osteogenic markers measured. At day 2, expression of all genes was similar between the surfaces examined, with the exception of OSX, which had higher expression on modSLA than TCPS (Fig. 5L). Osteogenic markers OSX (Fig. 5K) and RUNX2 (Fig. 5L) increased in HMSCs cultured on SLA and modSLA surfaces in comparison to TCPS and PT on day 4 and day 6, confirming osteogenic differentiation.

On day 4, expression of WNT3A (Fig. 5A) and CTNNB (Fig. 5C) was downregulated on SLA and modSLA surfaces in comparison to PT and TCPS. By day 6, expression of these two factors in cells on rough surfaces was less than 50% of the expression on the smooth surfaces, indicating downregulation of canonical Wnt signaling during osteoblastic differentiation. This was coupled with a downregulation in expression of the canonical receptor LRP5 on rough surfaces in comparison to TCPS (Fig. 5F). In contrast, expression of the non-canonical Wnt ligand WNT5a increased in cells grown on PT surfaces after 4 and 6 days in culture, but was greatly increased with time in cells on the rough SLA and modSLA substrates (Fig. 5B). This increased in expression over time was also seen in FZD2 (Fig. 5D) and FZD5 (Fig. 5E). Canonical Wnt inhibitors DKK1 (Fig. 5G) and DKK2 (Fig. 5H) increased with increasing surface roughness and hydrophilicity on day 4 and day 6. Expression of WIF1 increased in cells on rough surfaces at day 4 and day 6, but there was no effect of surface energy (Fig. 5I). However, CER1 was the same on all surfaces at day 4, and was only higher on rough surfaces in comparison to the smooth substrates at day 6 (Fig. 5J).

Integrins and Wnt Signaling

There was no change in integrin expression over time on TCPS (Fig. 5M–5R). Expression of ITGA1 was higher on SLA and modSLA surfaces than PT or TCPS at all time points (Fig. 5M). Expression on ITGA2 increased on PT at day 4 and 6 in comparison to TCPS, but was higher on SLA and modSLA surfaces at days 2, 4, and 6 in comparison to the smooth substrates (Fig. 5N). In contrast, ITGA5 expression was unchanged on TCPS and PT substrates (Fig. 5O) over time, but was downregulated on SLA and modSLA on day 4 and on modSLA on day 6 in comparison to TCPS and PT. ITGAV was highest on rough surfaces on day 4 and on Ti substrates than TCPS on day 6 (Fig. 5P). While cells on all substrates had similar expression of ITGB1 on day 2, expression was higher on rough SLA and modSLA in comparison to the smooth surfaces at day 4 and 6; ITGB1 expression did not change over time on TCPS or PT substrates (Fig. 5Q). There was no change in expression of ITGB3 in cells over time or between substrates (Fig. 5R).

To determine whether the increase in integrin expression was a result of increase in calcium-dependent Wnt signaling, we treated HMSCs with Wnt3a or Wnt5a. Treatment of HMSCs with Wnt5a increased expression of ITGA1 (Fig. 6A), ITGA2 (Fig. 6B), ITGAV (Fig. 6E), and ITGB1 (Fig. 6F) in comparison to control cultures. Expression of RUNX2 was regulated in a similar manner (Fig. 6H). Wnt3a treatment had no effect on the expression of any of these mRNAs. In contrast, Wnt3a treatment led to increased expression of ITGA3 (Fig. 6C), ITGA5 (Fig. 6D), and CTNNB (Fig. 6I) in comparison to control cultures, while Wnt5a had no effect. There was no change in ITGB3 in response to either treatment (Fig. 6G). Wnt5a increased mRNA for integrin subunits important in regulating cell response to surface microstructure and energy (ITGA1, ITGA2, ITGAV, and ITGB1), while Wnt3a had no effect. The increase in integrin expression was a result of an increase in calcium-dependent Wnt signaling.

Using WNT5A-silenced HMSCs (shWNT5A), we sought to determine whether Wnt5a regulated integrin expression in cells grown on microstructured Ti substrates. Real-time qPCR confirmed >90% knockdown of expression of WNT5A in shWNT5A-HMSCs (Fig. 6J). Expression of WNT5A increased in HMSCs in response to rough Ti substrates, but there was no change in expression in shWNT5A cells. While WNT3A expression decreased in HMSCs on Ti substrates, there was no change in expression in shWNT5A (Fig. 6K). Expression of ITGA1 increased with increasing surface roughness and energy in HMSCs, but in shWNT5A expression was unchanged compared to TCPS or PT (Fig. 6L). ITGA2 expression increased on SLA in comparison to TCPS and modSLA in comparison to the smooth substrates (Fig. 6M). However, ITGA2 expression was lower in shWNT5A cells was lower than HMSCs on all substrates and was not regulated by surface features. ITGA5 expression was downregulated in HMSCs on rough surfaces, but was unchanged in shWNT5A (Fig. 6O). HMSCs increased ITGAV on rough Ti substrates in comparison to smooth surfaces (Fig. 6P). WNT5A-silenced cells had increased ITGAV expression on TCPS, but decreased expression on SLA and modSLA substrates in comparison to TCPS. HMSCs showed increased ITGB1 expression on rough Ti substrates in comparison to smooth TCPS and PT (Fig. 6Q). Expression of ITGB1 was lower on Ti substrates in shWNT5A cells than HMSCs, but cells on modSLA had increased expression in comparison to TCPS or PT. Expression of ITGA3 (Fig. 6N) and ITGB3 (Fig. 6R) was not regulated by surface microstructure or energy in either HMSCs or shWNT5A.

DISCUSSION

Members of the Wnt family are known to affect osteoblast commitment, differentiation, and maturation as well as bone formation and development [33–36]. Studies from our laboratory have shown that Wnt signaling is regulated during osteoblast maturation on microstructured

Ti substrates [37]. However, the contribution of Wnt signaling in the differentiation of HMSCs on microstructured Ti surfaces is unknown. This study presents the separate contributions of Wnt3a and Wnt5a in the differentiation of HMSCs grown on complex microstructured titanium surfaces and the modulation in Wnt signaling that takes place in the early differentiation of HMSCs on these surfaces. Importantly, these results show that Wnt5a mediates osteoblastic differentiation on microtextured Ti via non-canonical calcium-dependent signaling. Moreover, Wnt5a modulates the expression of integrin subunits shown previously to mediate the effects of surface microtopography and energy.

Canonical Wnt Signaling

It has been suggested that Wnt3a increases HMSC proliferation and promotes stem cell renewal by activation of the Wnt/ β -catenin pathway [26, 38]. Our results support this hypothesis, showing that exogenous Wnt3a treatment increased HMSC cell number on smooth surfaces. However, it is important to note that Wnt3a had no effect on rough surfaces. We have shown that HMSCs on rough surfaces are committed towards an osteoblast phenotype both directly and indirectly, reducing HMSC number and increasing osteogenic markers on these surfaces [13]. The increase in cell number on smooth surfaces after Wnt3a treatment may be due to the less differentiated phenotype that these cells present; however, treated cells grown on microstructured surfaces showed similar behavior to cells treated with control or blocking antibody. Cell number was not affected after blocking endogenous Wnt3a on any of the tested surfaces, which suggests that Wnt3a has no effect on proliferation of more differentiated cells. HMSCs treated with exogenous Wnt3a increased alkaline phosphatase activity and decreased osteocalcin levels, but blocking endogenous Wnt3a with antibodies had no effect on alkaline phosphatase activity and increased osteocalcin levels. Although several studies have shown that Wnt3a induces alkaline phosphatase activity and increases bone sialoprotein and osteocalcin expression [39], it has also been reported that Wnt3a suppresses osteogenic differentiation in adult HMSCs, indicated by increased alkaline phosphatase activity coupled with decreases in osteocalcin and osteopontin levels [26, 40].

In addition to alkaline phosphatase activity and osteocalcin production, cells must regulate their microenvironment to support bone formation around the implant. Our studies have shown that osteoblast-like cells and HMSCs grown on microstructured Ti surfaces, produce an osteogenic environment increasing levels of OPG, TGF- β 1, VEGF, PGE2, Dkk1, and Dkk2 [13, 18]. In this study, exogenous Wnt3a treatment decreased OPG, increased VEGF-A and latent TGF- β 1 on all titanium surfaces, and had no effect on BMP2 or BMP4 levels. Blocking endogenous Wnt3a with antibodies decreased levels of VEGF with no effect on the other factors measured. These results confirm the hypothesis that Wnt3a negatively regulates osteoblastogenesis, supporting the idea that the key role of Wnt3a is in the self-renewal of HMSC population and in maintenance of the less differentiated niche.

Little is known about the influence of Wnt3a in the osteogenic microenvironment. Some studies have shown evidence that Wnt3a regulates VEGF expression in several cell types derived from mesenchymal origin [41, 42]. One proposed mechanism for this is the seven binding sites for β -catenin/TCF presented on the VEGF promoter [19]. Another study proposed that TGF- β 1 expression increased after Wnt3a treatment and the resulting TGF- β 1 increased VEGF expression [41]. Interestingly, in our study we found that both VEGF and latent TGF- β 1 increased with Wnt3a treatment, suggesting that both mechanisms are activated on microstructured Ti surfaces. Addition of exogenous Wnt3a or blocking of endogenous Wnt3a had no effect on BMP2 or BMP4 levels, suggesting that Wnt/ β -catenin does not increase osteogenesis, but may suppress osteoblast differentiation. Some reports showed that Wnt3a strongly induces the expression of Gremlin2 and Follistatin, potent

BMPs inhibitors [43]. Taken together, our data suggest that Wnt3a preserves a less mature phenotype of HMSCs grown on microstructured Ti surfaces.

Calcium-dependent Wnt Signaling

The non-canonical pathway activator Wnt5a had distinctly different effects on cells cultured on TCPS versus cells cultured on the Ti substrates. Wnt5a increased HMSC cell number on TCPS, had no effect on PT, and decreased HMSCs on rough surfaces. Blocking Wnt5a increased cell number on all titanium surfaces. While several studies have addressed the effect of Wnt3a in HMSCs proliferation and self-renewal, the effect of Wnt5a in HMSCs remains unclear. Some studies report that Wnt5a has no effect on HMSC proliferation, but exerts its effect in the commitment of HMSCs to an osteoblastic lineage, which also implicates a decrease in cell proliferation as shown in our study [25].

The hypothesis that Wnt5a increases commitment of HMSCs to an osteogenic lineage is supported by the increase in alkaline phosphatase specific activity and osteocalcin after Wnt5a exogenous treatment. We found previously that these two markers of osteoblast differentiation increased HMSCs grown on microstructured titanium surfaces [13]. This effect is more robust with the combination of microstructured surfaces and exogenous Wnt5a and decreased when endogenous Wnt5a is blocked. These results indicate an increase in osteoblast differentiation on rough surfaces, which is enhanced by exogenous Wnt5a. Unfortunately, the role of Wnt5a in osteoblast differentiation and bone formation remains unclear; however, the few studies focused on this molecule support the idea that Wnt5a increases osteoblast differentiation and osteogenic formation *ex vivo* [26]. These studies showed that Wnt5a increased alkaline phosphatase activity and osteocalcin transcriptional activity. Other studies have shown that Wnt5a inhibits commitment of HMSCs towards an adipogenic lineage and enhanced osteoblastogenesis [44]. It has been also observed that Wnt5a suppresses the Wnt/ β -catenin pathway and possibly induces Runx2 expression, which may explain the osteogenic potential of this molecule [24].

In this study, exogenous Wnt5a increased BMP2, BMP4, and latent TGF- β 1 on all Ti surfaces tested and had no effect on OPG and VEGF levels. The inhibition of endogenous Wnt5a decreased levels of BMP2 on the rough Ti surfaces but had no significant effect on the other parameters measured. The increase in BMP2, BMP4, and latent TGF- β 1 may be explained by the more differentiated phenotype that HMSCs adopt on microstructured Ti surfaces, and exogenous Wnt5a may enhance this effect, suggesting a robust activity of Wnt5a in osteoblast precursors or mesenchymal stem cells already committed towards an osteoblastic lineage. This hypothesis is supported by the minimal or no effect of Wnt5a in HMSCs grown on TCPS. To date, there is no evidence that Wnt5a induces BMP2 or BMP4 expression, but there is enough evidence demonstrating the ability of Wnt5a to promote osteogenesis. The essential role of Wnt5a was shown in the Wnt5a $^{-/-}$ mice model, where mice presented shortened limbs, absence of distal digits, and deformity in the developing face [45]. Furthermore, the overlapped expression pattern of Wnt5a and Runx2 in bone formation, the reduced expression of Runx2, and the absence of osteocalcin expression in Wnt5a $^{-/-}$ mice suggests a strong osteogenic activity of Wnt5a [46, 47]. One study suggested that Wnt5a is involved in osteogenic differentiation on titanium surfaces; however, this study focused only in gene expression and the increase in Wnt5a expression was just observed at one time point from the four measured [48].

Wnt Signaling Modulates Integrin Expression

Interestingly, our study showed a time-course correlation between increased expression of ITGA1, ITGA2, ITGAV, and ITGB1 and increased WNT5A expression. Integrins have been widely associated to early cell-biomaterial interactions such as cell attachment and

roughness recognition, but also they have been associated to osteoblast differentiation [8]. The integrin $\alpha 2\beta 1$ heterodimer recognizes collagen type I, the major organic component in bone, and some studies have shown that this integrin heterodimer is fundamental for osteoblast differentiation and maturation. Previously we determined ITGA2 and ITGB1 were up-regulated in HMSCs cultured on titanium surfaces with complex microstructure in comparison to smooth titanium or TCPS [13]. Knockdown of the $\beta 1$ integrin subunit impairs osteoblast recognition of complex microstructured titanium surfaces [9], and knockdown of the $\alpha 2$ integrin subunit renders the cells unable to produce an osteogenic environment [8]. Interestingly, Wnt5a treatment increased ITGA2 and ITGB1 expression, suggesting a possible role of Wnt5a in cell-extracellular matrix interactions. Studies in breast cancer cells indicated that treatment with Wnt5a or Wnt5a peptide up-regulated genes for collagen receptors discoidin receptor 1 (DDR1) and DDR2 [49, 50]. Wnt5a reduction has been found in breast cancer cells, and the reduction of Wnt5a induced a reduction of these collagen receptors, however exogenous treatment with Wnt5a protein or peptide increased levels of DDR1 and DDR2, as well as estrogen receptor alpha, leading to a decrease in metastatic cells [49, 50]. These studies indicate the importance of Wnt5a in cell attachment and cell-collagen interactions. Taken together with our results, this suggests that Wnt5a could have a positive feedback with integrins, particularly those associated with collagen type I. Interestingly, another integrin that also recognizes type I collagen, ITGA1, increases after Wnt5a treatment, emphasizing our hypothesis that Wnt5a modulates collagen type I receptors.

Our study highlights the importance of physiologically relevant culture substrates in elucidating signaling pathways. For most of the factors examined, we saw no difference between treated and untreated cells on TCPS. It was only when the cells were cultured on substrates similar to topographies seen in the body were the contributions of the signaling pathway apparent.

CONCLUSIONS

These results indicate that Wnt5a affects differentiation of HMSCs grown on microstructured titanium surfaces. Additionally our data suggest that this effect may be mediated through local factors, particularly through BMPs. The effect on OPG levels suggests that Wnt5a is important in regulation of bone turnover, particularly on rough surfaces. Collectively, our results show evidence that osseointegration requires Wnt signaling from both pathways explored in this study and that these pathways are regulated in an autocrine and paracrine fashion on microstructured titanium surfaces. Wnt activators, inhibitors, and receptors may play an important role around implants and Wnt signaling pathways must be tightly regulated for successful osteoblast differentiation, maturation, and eventual bone formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of microstructured Ti substrates. Pretreatment (PT), sandblasted/acid-etched (SLA), or hydrophilic SLA (modSLA) substrates were examined by scanning electron microscopy (SEM, A) at 1.0K $\times$ (upper panels, scale bar 10 μ m) or 30.0K $\times$ (lower panels, scale bar 300 nm). Surface chemical composition was assessed using XPS (B). High resolution spectra of O, Ti, and C are shown in insets. Surface energy was assessed by contact angle (C). Surface roughness and morphology were assessed using confocal scanning optical microscopy (CSOM, D). Positive (upper panels) and negative (lower panels) representations of the surfaces are shown.

Figure 2.

Effect of surface microstructure and surface energy on HMSC gene expression. HMSCs were plated on TCPS or Ti substrates and cultured until confluence on TCPS. Cells were incubated with fresh media for 12 hours, RNA extracted, and real-time qPCR performed on cDNA templates to measure mRNA levels of WNT1 (A), WNT3A (B), WNT5A (C), WNT7B (D), CTNNB (E), AXIN2 (F), FZD1 (G), FZD2 (H), FZD3 (I), FZD4 (J), FZD5 (K), FZD6 (L), FZD7 (M), FZD8 (N), and FZD9 (O). * $p < 0.05$, Ti v. TCPS; # $p < 0.05$, v. PT.

Figure 3.

Effect of Wnt3a on HMSC response to surface microstructure and surface energy. HMSCs plated on TCPS, PT, SLA, or modSLA surfaces were cultured with 50ng/mL Wnt3a or 1:200 AbWnt3a until confluence. At confluence, cell number (A), alkaline phosphatase specific activity (B), and levels of secreted osteocalcin (C), OPG (D), BMP2 (E), BMP4 (F), VEGF (G), and latent TGF-β1 (H) were determined. *p<0.05, Ti v. TCPS; #p<0.05, treatment with either Wnt3a or AbWnt3a v. untreated HMSCs on each surface.

Figure 4.

Effect of Wnt5a on HMSC response to surface microstructure and surface energy. HMSCs plated on TCPS, PT, SLA, or modSLA surfaces were cultured with 250ng/mL Wnt5a or 1:200 AbWnt5a until confluence. At confluence, cell number (A), alkaline phosphatase specific activity (B), and levels of secreted osteocalcin (C), OPG (D), BMP2 (E), BMP4 (F), VEGF (G), and latent TGF-β1 (H) were determined. *p<0.05, Ti v. TCPS; #p<0.05, treatment with either Wnt5a or AbWnt5a v. untreated HMSCs on each surface.

Figure 5.

Time course of expression in HMSCs grown on microstructured Ti surfaces. HMSCs were plated on TCPS, PT, SLA, or modSLA surfaces. Cells were harvested two (white bar), four (grey bar), or six (black bar) days after plating. RNA was extracted and real-time qPCR performed on cDNA templates to measure mRNA levels of WNT3A (A), WNT5A (B), CTNNB (C), FZD2 (D), FZD5 (E), LRP5 (F), DKK1 (G), DKK2 (H), WIF1 (I), CER1 (J), RUNX2 (K), OSX (L), ITGA1 (M), ITGA2 (N), ITGA5 (O), ITGAV (P), ITGB1 (Q), and ITGB3 (R). * $p < 0.05$, Ti v. TCPS; # $p < 0.05$, v. PT; \$ $p < 0.05$, v. SLA for each time point.

Figure 6.

Effect of Wnt signaling on integrin expression on microstructured Ti surfaces. I) HMSCs were plated on TCPS or Ti substrates and cultured until confluence on TCPS. Cells were treated with 50ng/ml Wnt3a or Wnt5a for 12 hours and expression of ITGA1 (A), ITGA2 (B), ITGA3 (C), ITGA5 (D), ITGAV (E), ITGB1 (F), ITGB3 (G), RUNX2 (H), and CTNNB (I) measured by real-time qPCR. * $p < 0.05$, v. control; # $p < 0.05$, v. Wnt3a-treated HMSCs. II) HMSCs (WT, black bar) and HMSCs with silenced Wnt5a (shWNT5A, white bar) were plated on TCPS or Ti substrates and cultured until confluence on TCPS. Cells were incubated with fresh media for 12 hours, RNA extracted, and real-time qPCR performed on cDNA templates to measure mRNA levels of WNT5A (J), WNT3A (K), ITGA1 (L), ITGA2 (M), ITGA3 (N), ITGA5 (O), ITGAV (P), ITGB1 (Q), and ITGB3 (R). * $p < 0.05$, Ti v. TCPS; # $p < 0.05$, v. PT; \$ $p < 0.05$, v. SLA; @ $p < 0.05$, shWNT5A vs. WT.