Published in final edited form as:
Biomaterials. 2010 March ; 31(8): 2015–2024. doi:10.1016/j.biomaterials.2009.11.071.

The roles of Wnt signaling modulators Dickkopf-1 (Dkk1) and Dickkopf-2 (Dkk2) and cell maturation state in osteogenesis on microstructured titanium surfaces

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Abstract

Osteoblast differentiation on tissue culture polystyrene (TCPS) requires Wnt/beta-catenin signaling, regulating modulators of the Wnt pathway like Dickkopf-1 (Dkk1) and Dkk2. Osteoblast differentiation is increased on microstructured titanium (Ti) surfaces compared to TCPS; therefore, we hypothesized that surface topography and hydrophilicity affect Dkk1 and Dkk2 expression and that their roles in osteoblast differentiation on Ti differs depending on cell maturation state. Human osteoblast-like MG63 cells, normal human osteoblasts (HOBs), and human mesenchymal stem cells (MSCs), as well as MG63 cells stably silenced for Dkk1 or Dkk2 were grown for 6 days on TCPS and Ti surfaces (PT [Ra<0.2 μm], SLA [Ra = 4 μm], modSLA [hydrophilic-SLA]). Dkk1 and Dkk2 mRNA and protein increased on SLA and modSLA for all cell types, but exogenous rhDkk1 and rhDkk2 affected MSCs differently than MG63 cells and HOBs. Silencing Dkk1 reduced MG63 cell number on TCPS and PT, but increased differentiation on these substrates. Silencing Dkk2 reduced stimulatory effects of SLA and modSLA on osteoblast differentiation; Dkk2 but not Dkk1 restored these effects. Antibodies to Dkk1 or Dkk2 specifically blocked substrate-dependent changes caused by the proteins, demonstrating their autocrine action. This indicates major roles for Dkk1 and the canonical Wnt pathway in early-stage differentiation, and for Dkk2 and Wnt/Ca^{2+}-dependent signaling in late-stage differentiation on microstructured and hydrophilic surfaces, during osseointegration.

Keywords

Osseointegration; Titanium; Osteoblast; Mesenchymal stem cell; Surface roughness; Cell signaling

1. Introduction

Bone substitutes and metallic implants are widely used in order to facilitate or enhance bone healing, regeneration and osseointegration. In the United States, nearly 13 million people suffer from bone fractures yearly, and dental and orthopaedic implants are used routinely to

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Appendix. Supplementary data Note: Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.biomaterials.2009.11.071.

restore function. One of the most frequently used materials for implants is titanium (Ti); however, most cell culture experiments concerning osteoblast differentiation have been performed using tissue culture polystyrene (TCPS) as the substrate. As a result, the mechanisms by which cells differentiate, mature, and produce bone on these biomaterials are less well understood.

Genetic evidence in frogs, mice, and humans indicates that Wnt signaling is required for embryonic bone development [1,2], but the role of Wnt signaling in bone healing, regeneration and osseointegration is not clear. In the canonical Wnt pathway, a Wnt protein binds a Frizzled (Fzd) receptor and a co-receptor (LDL receptor-related protein [Lrp5 or Lrp6]), resulting in Dishevelled activation and inhibition of the Axin/GSK3β/APC complex. When this occurs, GSK3β is unable to phosphorylate β-catenin and instead, non-phosphorylated β-catenin accumulates in the cytoplasm, translocates into the nucleus, and modulates gene transcription [1].

A large number of antagonists modulate the Wnt signaling pathway. These include the Dickkopf (Dkk) family, Wnt inhibitory factor, Frizzled-related proteins (FRP), and Cerberus and Sclerostin families. Both Dkk1 and Dkk2 inhibit the canonical Wnt signaling pathway. They bind to Lrp5/6, preventing formation of the Wnt-Fzd-Lrp complex [3–5]. Both proteins modulate skeletal development, but they do not appear to have the same effect on cells or act through the same mechanisms. Dkk1−/− mice have morphogenetic defects during development of their head and limbs, resulting in embryologic lethality [6]. Dkk2−/− mice are osteopenic and osteoblasts from these mice exhibit impaired function [3], producing increased levels of RANK ligand but not osteoprotegerin (OPG). These observations suggest that Dkk1 and Dkk2 both mediate osteoblast maturation and tightly regulate bone formation, but their effects occur at different stages of maturation. Although both Dkk1 and Dkk2 are potent Wnt inhibitors, Dkk2 could have a role in osteoblast maturation through an indirect mechanism. This is supported by evidence showing that Dkk2 can also activate, rather that inhibit, the canonical Wnt pathway [3,7].

In vivo, osteoblasts colonize and differentiate on bone surfaces that have been preconditioned by osteoclasts, resulting in a microstructure [8] and chemistry [9] very different from that of TCPS. Studies using microstructured Ti and Ti6Al4V substrates with topographies similar to osteoclast resorption pits indicate that osteoblast-like MG63 cells, normal human osteoblasts (HOBs), fetal rat calvarial cells, and neonatal mouse osteoblasts exhibit a more differentiated phenotype than when they are cultured on TCPS or smooth Ti substrates [10]. Moreover, they respond to exogenous regulatory factors more robustly than when they are grown on TCPS, including estrogen [11], 1,25-dihydroxyvitamin D3 [1α, $25(OH)_2D_3$] [12], and rhBMP-2 [13]. The osteoblastic phenotype, which is characterized by osteocalcin and osteoprotegerin (OPG) production, is further enhanced when these cells are grown on hydrophilic Ti substrates with complex micron scale and submicron scale topography [14]. This substrate dependent difference in osteoblast behavior on materials other than TCPS raises the possibility that Wnt signaling may also be affected.

The aim of the present study was to evaluate the effect of surface microtopography and chemistry on the regulation of the Wnt antagonists Dkk1 and Dkk2. We hypothesized that Dkk1 and Dkk2 act at different stages of osteoblast maturation. To test this, we compared the effects of Dkk1 and Dkk2 on HOB cells to those of human mesenchymal stem cells (MSCs) cultured on smooth and microstructured Ti substrates. To better understand the mechanism by which Dkk1 and Dkk2 mediate their effects on osteoblast differentiation, we developed human osteoblast-like MG63 cell lines stably silenced for expression of each protein. The results of the present study begin to elucidate the mechanisms of implant osseointegration and provide information that can be used to improve these processes.

2. Materials and methods

2.1. Cell culture

Ti disks were prepared from 1 mm thick sheets of grade 2 unalloyed Ti (ASTM F67 "Unalloyed Ti for surgical implant applications") and supplied to us by Institut Straumann AG (Basel, Switzerland). The disks were punched to be 15 mm in diameter so as to fit snugly into the well of a 24-well tissue culture plate. The fabrication methods and resulting morphology of the substrates have been published previously [14,15]. Briefly, the pretreatment (PT) surface is a smooth Ti surface with mean peak to valley roughness (R_a) of 40 nm. PT surfaces that have been grit blasted and acid etched exhibit craters approximately 100 μm in diameter that are overlaid with pits 1–3 μm in diameter, which are coated with spikes approximately 700 nm in height, resulting in Ra ~3.2 μm (SLA). SLA surfaces were also processed so that exposure to ambient atmosphere was minimized, retaining the hydrophilic chemistry of a clean TiO2 surface (modSLA). modSLA surfaces were rinsed under nitrogen protection to prevent exposure to air during the fabrication procedure, and then stored in a sealed glass tube containing isotonic NaCl solution. These sealed disks were sterilized by gamma irradiation at 25 kGy over night. SLA and modSLA disks have identical complex micron scale and submicron scale structural features but different surface chemistries. The modification process changes surface chemical composition by decreasing carbon contamination more than 50%, thereby retaining a higher surface energy. Advancing contact angles were used as an indicator for calculating surface free energy. SLA is very hydrophobic with a water contact angle of 139.9°; in contrast, the contact angle of modSLA is close to 0°, indicating a very hydrophilic surface. In vivo, bone-to-implant contact is greater around SLA surfaces than PT, and the rate of osteogenesis is further increased around modSLA than around SLA implants [16].

Human osteoblasts (HOBs) were isolated from bone obtained from Children's Healthcare of Atlanta under Institutional Review Board approval. Bone fragments were washed in Dulbecco's Modification of Eagle's Medium (DMEM) containing 3% penicillin-streptomycin, followed by incubation in trypsin-EDTA (Invitrogen, Carlsbad, CA) for 1h. The bone fragments were cut into 1 mm^2 pieces, which were cultured in a 100 × 20 mm Petrie dish for two weeks, permitting immature osteoblasts to migrate onto the culture dish surface. Human MSCs were purchased from Lonza (Walkersville, MD) and grown in mesenchymal stem cell growth medium (Lonza). Human MG63 osteoblast-like osteosarcoma cells were obtained from the American Type Culture Collection (Rockville, MD). These cells have been well characterized and exhibit increased alkaline phosphatase activity and osteocalcin synthesis in response to 1α,25$(OH)_2D_3$. Osteoblastic differentiation of MG63 cells increases when they are cultured on Ti substrates of increasing micron scale and submicron scale roughness [17] and surface energy [14]. The cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were cultured at 37 °C and 100% humidity.

2.2. Dkk1 and Dkk2 protein levels

Cells were plated on TCPS, PT, SLA or modSLA surfaces at a density of 5000 cells/cm^2 for MSCs or 10,000 cells/cm^2 for HOB and MG63 cells. Media were changed after 24h and every 48h until cells reached confluence on TCPS (about 7 days). At confluence, the media were changed and cells incubated for 24h. After incubation, the conditioned media were collected and used to measure levels of Dkk1 and Dkk2. Dkk1 levels were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN). Dkk2 levels were analyzed by standard ELISA technique using a mouse monoclonal Dkk2 antibody (ab19024) as capture antibody and a goat polyclonal Dkk2 antibody (ab21187) as detection antibody (Abcam, Cambridge, MA).

2.3. Dkk1 and Dkk2 silencing

To assess the role of Dkk1 and Dkk2 in osteoblast response to surface microstructure and surface energy, we used shRNA technology to silence endogenous expression. For these experiments, MG63 cells were transduced with empty vector or one of four different shRNA lentiviral transduction particles (Mission®, Sigma Aldrich, St. Louis, MO). MG63 cells were plated at 20,000 cells/cm^2 and cultured overnight as above. Particles were added to the cells at a multiplicity of infection of 7.5 and incubated for 18h. After incubation, transduced cells were selected with 0.25 μg/ml of puromycin. Dkk1 and Dkk2 expression and levels were assessed by RT-PCR and Western blot for the respective cell lines.

Stably silenced MG63 cells lines were established for Dkk1 and Dkk2. Three different sequences were used to silence Dkk1. Cells transduced with clone 33384 exhibited a greater than 90% reduction in Dkk1 mRNA by Real-time PCR (Supplemental Fig. 1A) and a greater than 70% reduction in Dkk1 protein level by Western blot analysis (Supplemental Fig. 1B). Two cell lines exhibited a greater than 70% reduction in Dkk2 protein (Supplemental Fig. 2B), but only one of these, clone 33389, also showed a greater than 70% decrease in mRNA (Supplemental Fig. 2A). Accordingly, cells transduced with these clones were selected for subsequent studies with Dkk1- and Dkk2-silenced cells, respectively.

Silenced cells and MG63 cells were plated on all surfaces and cultured as above. Wild type and silenced MG63 cells were treated daily with 1 μg/mL recombinant human Dkk1 (rhDkk1) or rhDkk2 or with bovine serum albumin until confluent. In addition, wild type MG63 cell cultures were treated with rhDkk1 or Dkk2 in the presence of non-specific IgG or an antibody to Dkk1 (MAB1096, R&D Systems) or antibody to Dkk2 (ab21187, Abcam). At confluence, osteocalcin, osteoprotegerin (OPG), active and latent transforming growth factor beta-1 (TGF-β1) and vascular endothelial growth factor A (VEGF-A) were measured in the conditioned media as described below.

2.4. Effect of exogenous Dkk1 and Dkk2 on MSCs

To determine the effect that Dkk1 and Dkk2 have on osteoblastic differentiation of multipotent cells, MSCs were cultured as described above on TCPS, PT, SLA, and modSLA surfaces. Cells were treated everyday with 1.0 μg/ml rhDkk1 or rhDkk2 or with bovine serum albumin [4,18] until confluent. At confluence, cells were harvested and conditioned media collected as described below.

2.5. Assays of cell response

Total cell number was determined and alkaline phosphatase specific activity of cell lysates were assayed as described previously [11]. Levels of osteocalcin (Human Osteocalcin RIA kit, Biomedical Technologies, Inc., Stoughton, MA), OPG, active and latent TGF-β1, and VEGF-A (DuoSet ELISA kits, R&D Systems, Inc., Minneapolis, MN) levels were measured. To differentiate between active and latent TGF-β1, an aliquot of the conditioned media was assayed directly without acid-activation (active TGF-β1); a second aliquot was activated, providing the level of total TGF-β1. Latent TGF-β1 was calculated by subtracting the amount of active growth factor from the total.

2.6. Statistical analysis

Data presented are from one of two sets of experiments, with comparable results. Each data point is the mean ± SEM for six independent cultures. Data were analyzed by analysis of variance and significant differences between groups determined using Bonferroni's modification of the Student's *t*-test. $P \leq 0.05$ was considered to be significant.

3. Results

3.1. Effects of substrate microstructure on Dkk1 and Dkk2 expression

HOB cells, MG63 cells, and MSCs all produced Dkk1 and Dkk2 protein and released them into their conditioned media in a substrate dependent manner. MSC and MG63 cells had higher levels of Dkk1 in conditioned media from cultures grown on SLA and modSLA in comparison to TCPS (Fig. 1 A, B). However, HOBs had higher levels of Dkk1 only on the rough, hydrophobic modSLA surfaces (Fig. 1C) in comparison to TCPS. In contrast, Dkk2 protein was slightly increased in MSCs grown on PT and SLA (Fig. 1D) and showed an almost 300% increase on modSLA (Fig. 1D). Dkk2 protein produced by MG63 cells was increased 200% on SLA and modSLA in comparison to TCPS or PT (Fig. 1E). Similarly, Dkk2 was increased in the media of HOBs grown on SLA and modSLA (Fig. 1F).

Changes in Dkk1 and Dkk2 protein reflected substrate-dependent differences in mRNA. MG63 cells had twice as much DKK1 mRNA when grown on SLA and modSLA than when they were on TCPS or PT (Supplemental Fig. 3A). Similarly, DKK2 expression increased 300% in MG63 cells grown on SLA and modSLA compared to TCPS (Supplemental Fig. 3B). MG63 cells had small but significant increases in DKK1 mRNA on SLA and modSLA (Supplemental Fig. 3C). However, in HOB cells DKK2 mRNA was increased more than 100% on SLA and 200% on modSLA (Supplemental Fig. 3D).

3.2. Dkk1 and Dkk2 act as autocrine mediators

3.2.1. Effect of Dkk1 and Dkk2 silencing in MG63 cells—Endogenous Dkk1 and Dkk2 were required for MG63 cell response to substrate microarchitecture and surface energy. Dkk1-silenced cells exhibited reduced cell numbers on TCPS, PT, and SLA in comparison with wild type MG63 cells, but Dkk1-silencing had no effect on modSLA (Fig. 2 A). Dkk1-silencing blocked the substrate dependent increase in alkaline phosphatase specific activity normally seen in cultures grown on modSLA (Fig. 2B). In contrast, Dkk1-silenced cells grown on TCPS, PT, and SLA had higher levels of osteocalcin and OPG in their media than wild type MG63 cells, levels that were comparable to those produced by wild type cells cultured on modSLA (Fig. 2 C, D). Interestingly, the increase in VEGF-A production seen in wild type cells with increasing surface roughness and surface energy was markedly reduced (Fig. 2E). Dkk1 silencing increased active and latent TGF-β1 on TCPS, PT, and SLA compared to wild type cells, but had no effect on production of either form of the growth factor in cells grown on modSLA (Fig. 2F).

Silencing Dkk2 resulted in very different responses to surface microstructure or surface energy. Reduced Dkk2 expression had no effect on cell number on any of the substrates (Fig. 3 A), but it inhibited differentiation typical of wild type MG63 cells grown on SLA and modSLA. Both alkaline phosphatase activity (Fig. 3B) and osteocalcin production (Fig. 3C) were reduced in the silenced cells. While the inhibitory effect of Dkk2 silencing on differentiation was limited to SLA and modSLA, the silenced cells exhibited reduced levels of factors involved in osteoblast-dependent regulation of osteoclast activity on all of the test surfaces. OPG production was reduced approximately 10% on TCPS and PT. The stimulatory effect of SLA on OPG was reduced by 20% and the effect of growth on modSLA was reduced by 40% (Fig. 3D). VEGF-A production was also sensitive to Dkk2 silencing and was reduced by 50% on all surfaces (Fig. 3E). Dkk2-silencing reduced latent and active TGF-β1 on all surfaces (Fig. 3F).

3.2.2. Addition of exogenous Dkk1 and Dkk2 to MG63 wild type and silenced cells—Exogenous Dkk1 slightly reduced wild type cell number on TCPS and smooth PT, but there was no change when added to wild type cells on the microstructured SLA and

modSLA surfaces (Fig. 4 A). Adding Dkk1 to the Dkk1-silenced cells partially restored the decrease in number seen on the smooth surfaces as shown in Fig. 2A. Exogenous Dkk1 reduced alkaline phosphatase activity of wild type MG63 cells grown on SLA and modSLA (Fig. 4B). When Dkk1-silenced cells were treated with exogenous Dkk1, enzyme activity was comparable to untreated wild type MG63 cells. Dkk1 had a similar effect on the levels of osteocalcin (Fig. 4C), VEGF (Fig. 4E), and total TGF-β1 (Fig. 4F) in the conditioned media of wild type and Dkk1-silenced MG63 cells. In contrast, Dkk1 treatment had no effect on OPG production in either MG63 or Dkk1-silenced cells (Fig. 4D).

Responses to exogenous Dkk2 were very different from those seen in response to Dkk1. Dkk2 slightly decreased cell number on TCPS but had no effect on cell number (Fig. 5 A) in wild type MG63 cell cultures grown on any of the other substrates examined. When Dkk2-silenced cells were treated with exogenous Dkk2, cell number on SLA and modSLA was reduced but there was no effect on TCPS or PT. Dkk2 stimulated alkaline phosphatase in wild type and Dkk2-silenced cells grown on TCPS and PT, but had no effect on enzyme activity in either cell type grown on SLA or modSLA (Fig. 5B). Osteocalcin levels were increased in wild type cells grown on TCPS and PT and in Dkk2-silenced cells grown on SLA and modSLA (Fig. 5C). Dkk2 caused a small increase in OPG production by wild type cells grown on TCPS and PT, but its effect on Dkk2-silenced cells was to reduce OPG by 50% on these same surfaces (Fig. 5D). Similarly, VEGF was increased in all wild type cultures treated with Dkk2 and reduced in Dkk2-silenced cells grown on TCPS, PT, and SLA (Fig. 5E). Exogenous Dkk2 stimulated TGF-β1 production in cultures grown on TCPS and PT but had no effect on Dkk2-silenced cells grown on the other surfaces (Fig. 5F).

The effects of Dkk1 and Dkk2 were mediated by separate mechanisms. Dkk2, but not Dkk1, was able to reverse the effects of Dkk2 silencing (Supplemental Fig. 4). Moreover, many of the responses to surface microstructure and surface energy seen in silenced cells were also seen in wild type MG63 cells treated with specific antibodies to Dkk1 (Supplemental Fig. 5) or Dkk2 (Supplemental Fig. 6).

3.3. Role of Dkk1 and Dkk2 in MSC differentiation

MG63 cells are useful in studies examining factors that stimulate osteoblastic differentiation because they represent a relatively immature phenotypic state in the osteoblast lineage [10]. In order to determine if Dkk1 or Dkk2 were acting to promote commitment of multipotent cells to the osteoblast lineage, we examined their effects on MSCs. Cell number was reduced in all cultures of MSCs grown on Ti substrates and this effect was greatest on the microstructured SLA and modSLA surfaces (Fig. 6A). Exogenous Dkk1 increased MSC number on all substrates (Fig. 6A). The increase in alkaline phosphatase specific activity seen with increasing surface roughness was decreased with exogenous Dkk1 (Fig. 6B), as were levels of osteocalcin (Fig. 6C), indicating a decrease in differentiation toward an osteoblast phenotype. Levels of OPG (Fig. 6D) and VEGF-A (Fig. 6E) decreased on all surfaces in response to treatment with Dkk1. Dkk1 did not affect production of active or latent TGF-β1 in MSC cultures grown on TCPS or PT, but it reduced both forms of the growth factor in cultures grown on SLA and modSLA (Fig. 6F).

Dkk2 had a similar effect on MSCs with respect to cell number (Fig. 7A), alkaline phosphatase specific activity (Fig. 7B), and osteocalcin production (Fig. 7C), but its effects were primarily on cells grown on SLA and modSLA. Whereas Dkk1 reduced OPG on PT, SLA and modSLA (Fig. 6D), Dkk2 reduced OPG only on the smooth TCPS and PT surfaces (Fig. 7D). Dkk2 reduced VEGF levels only on SLA and modSLA (Fig. 7E). Its effects on production of latent and active TGF-β1 were complex and substrate dependent. Dkk2 reduced latent TGF-β1 in MSC cultures grown on TCPS and SLA, and it reduced active TGF- β1 levels in cultures grown on SLA and modSLA (Fig. 7F).

4. Discussion

This study confirms that MG63 osteoblast-like cells exhibit similar responses to microstructured Ti substrates as normal human osteoblasts, providing further validation of the MG63 cell model. Others have observed that MG63 cells and HOB cells exhibit comparable cytokine production when grown on these substrates [19] and preclinical and clinical studies demonstrate that surface designs that promote MG63 cell differentiation in culture also promote increased bone-to-implant contact and greater pull-out strength in vivo [20–22]. The effect of the microstructured surface is to induce a shift to a mature osteoblast phenotype, which is due to an increasingly broad range of gene regulation, particularly evident in cells grown on complex microtopographies with hydrophilic chemistry.

Although these surface modifications have been applied to diverse dental and orthopaedic implants, significantly improving the patients' prognosis, healing and bone-to-implant contact, relatively little is known about the mechanisms involved. The present study implicates Dkk1 and Dkk2 in this process. Expression and actions of Dkk1 and Dkk2 are sensitive to surface microstructure commonly used on dental and orthopaedic implants. MSCs, HOBs and MG63 cells all expressed Dkk1 and Dkk2 in a substrate dependent manner with greater levels of mRNA and secreted protein in cultures grown on Ti surfaces with rough microtopographies. However, Dkk1 and Dkk2 played different roles on osteoblastic differentiation in each of these cell types.

Our results support the hypothesis that Dkk1 acts to prevent MSC differentiation, thereby conserving an undifferentiated stem cell phenotype [23]. While exogenous Dkk1 increased MSC cell number on all Ti surfaces, it reduced osteoblastic differentiation, as indicated by classic markers such as alkaline phosphatase activity and production of osteocalcin and OPG. Dkk2 affected MSC proliferation and differentiation on microstructured surfaces in a similar manner to Dkk1. Others have shown that Dkk2 is a potent Wnt/β-catenin antagonist in MSCs [4], but its specific role in this inhibition is not clear.

Our results also show that Dkk1 and Dkk2 are involved in the autocrine regulation of committed osteoblasts. MG63 cells responded to exogenous Dkk1 and Dkk2, indicating that they expressed functional receptors. However, the results suggest that Dkk1 and Dkk2 act in different ways to modulate proliferation and differentiation. Their effects were substrate dependent: Dkk1 affected cells grown on the microstructured and high energy SLA and modSLA surfaces, whereas Dkk2 acted on the cells grown on smooth TCPS and PT surfaces. This suggests that each factor acted on a different population of cells within the osteoblast lineage, based on the observation that MG63 cells express a more differentiated phenotype on SLA and modSLA than on TCPS or PT substrates. Further evidence that the effects of Dkk1 and Dkk2 on the MG63 cells were specific, was provided by the fact that antibodies to Dkk1 blocked only responses to exogenous Dkk1 whereas antibodies to Dkk2 blocked only responses to Dkk2. Moreover, only Dkk2 could rescue the effects of Dkk2 silencing, supporting the hypothesis that the two proteins regulated different responses in the cells.

The results of experiments in which Dkk1 or Dkk2 expression was silenced, showed the importance of endogenous synthesis of the two factors to the downstream release of autocrine and paracrine factors that modulate bone formation and remodeling. Knockdown of Dkk1 reduced cell number and increased osteocalcin and osteoprotegerin levels in a substrate dependent manner. Moreover, secreted levels of VEGF-A were dramatically decreased. In contrast, Dkk2-silencing reduced production of factors associated with autocrine regulation of osteoblast differentiation (TGF-β1) as well as regulation of

osteoclast activity (OPG, TGF-β1), all of which are elevated in wild type MG63 cell cultures grown on SLA and modSLA compared to TCPS or the smooth PT surface [14,24].

Our in vitro observations using shRNA support studies using calvarial osteoblasts from $Dkk1^{+/-}$ and $Dkk1^{+/+}$ mice [3]. $Dkk1^{+/-}$ osteoblasts proliferate more than osteoblasts from $Dkk1^{+/+}$ animals. Moreover, $Dkk1^{+/-}$ osteoblasts exhibit a 2-fold greater response to Wnt3a than wild type cells and, when treated with BMP2, are 2–7 times more responsive than $Dkk^{+/+}$ cells. This suggests that the higher bone mass present in $Dkk1^{+/-}$ mice compared to wild type mice is due to greater numbers of immature osteoblasts.

Interestingly, Dkk2 silencing did not affect cell number on any of the surfaces used in this study. Similar results were found in the Dkk2 deficient model in which mice surprisingly present osteopenia due to major defects in mineralization, including reduced osteocalcin, but without change in osteoblast number [3]. Similarly, we found that Dkk2-silencing caused a decrease in osteocalcin production by MG63 cells. It is likely that the failure to express osteocalcin is secondary to the failure of the osteoblasts to progress in their lineage to a terminal differentiation state in the absence of Dkk2. Support for this hypothesis was provided by the observation that Dkk2-silenced KS483 cells exhibit decreased mineralized nodule formation [4].

Preclinical and clinical studies have shown that microstructured implant surfaces support greater bone-to-implant contact than smooth surfaced implants [25,26], suggesting that factors are released during osseointegration that not only promote osteogenesis but result in net bone anabolism. Importantly for periimplant bone formation, Dkk2-silencing reduced production of local factors like TGF-β1 and OPG, which control bone remodeling by modulating osteoclast activity [27,28]. These results are in agreement with reports showing that Dkk2-null mice exhibited increased RANKL, but no change in OPG [3]. Taken together, the results support the hypothesis that Dkk2 secreted by osteoblasts on microstructured Ti substrates acts in an autocrine manner to mediate the expression and/or activation of TGF-β1, with downstream increases in OPG.

Dkk treatment of wild type MG63 cells also showed that the two proteins mediate different pathways when the cells are grown on Ti substrates rather than TCPS. Exogenous rhDkk1 had no effect on MG63 cell number. This was unexpected based on studies using 2.3-*Dkk1*-transgenic mice, in which Dkk1 is overexpressed, resulting in a depletion of osteoblasts and an osteopenic phenotype [29]. Our results suggest that the depletion of osteoblasts observed in 2.3-*Dkk1*-transgenic mice is due to a reduction in osteoblastic differentiation, specifically of more mature osteoblasts. The inhibitory effects of Dkk1 on MG63 cell differentiation were most evident in cultures grown on microstructured Ti, and these substrates support a more mature phenotype than either TCPS or PT surfaces [30,31]. In vitro studies using Dkk1 over-expressing cells show that Dkk1 reduces alkaline phosphatase activity but does not alter Runx2, osterix, or osteocalcin [3]. This suggests that Dkk1 mainly affects the function of mature osteoblasts and the commitment of pluripotent cells to an osteoblast linage.

Taken together, our results show that Dkk1 affects the commitment of multipotent cells and the function of mature osteoblasts as we could rescue the mature phenotype with exogenous Dkk1. Our findings indicate that Dkk1 has pleiotropic effects, which are dependent on the state of cell maturation in the osteoblast lineage. Others have also observed differential effects of Dkk1. 1,25-dihydroxy vitamin D3 [$1\alpha,25(OH)_2D_3$] has been shown to suppress Dkk1 in mouse marrow stromal cells [32] and to inhibit osteocalcin expression in the pre-osteoblast mouse MC3T3-E1 cells [33]. In contrast, $1\alpha,25(OH)_2D_3$ increases osteocalcin expression in mature HOB cells, mouse calvarial osteoblasts, and rat osteoblasts, as well as

MG63 cells, and these effects are greatest when the cells are cultured on microstructured Ti [12,34]. While it is possible that differences in cell source are responsible for these differences in response, it is more likely that they are due to differences in maturation state and that Dkk1 plays a role. Additional evidence is the observation that cells isolated from multiple myeloma present high levels of Dkk1 and these myeloma cells inhibit bone formation by inhibiting osteoblast differentiation [35]. Moreover, Dkk1 significantly blocks BMP2 induced mineralization [36]. Finally, our study showed that exogenous Dkk1 rescued the mature osteoblast phenotype in Dkk-1 silenced cells grown on microstructured Ti, but did not affect proliferation.

Whereas Dkk1 had its greatest effects on cells grown on microstructured Ti, exogenous Dkk2 had its predominant effects in MG63 cells grown on smooth TCPS and PT substrates, increasing alkaline phosphatase activity, osteocalcin, OPG, VEGF and TGF- β1. Interestingly, endogenous Dkk2 was elevated on the microstructured SLA and modSLA, suggesting the possibility that Dkk2 is needed for the shift to a more differentiated phenotype. Silencing experiments confirmed this hypothesis. In addition, transduction with Dkk2-expressing adenovirus-mediated vectors in calvarial osteoblasts induced a mineralizing phenotype, increasing mineral deposits with a mineral-matrix ratio similar than control osteoblast [3]. Dkk2 may have a dual role in osteoblast differentiation, acting like an antagonist of Wnt canonical signaling in early stages of osteoblast differentiation and as an agonist at later stages. In *Xenopus* embryos, Dkk2 can weakly activate Wnt/β-catenin signaling when overexpressed and in presence of overexpressed LRP6, the Dkk2 cysteine-rich domain can activate rather than inhibit Wnt signaling in human 293 fibroblasts [4].

The present study also shows that surface roughness regulates the production of VEGF-A, which stimulates angiogenesis and maturation of blood vessels [37]. The production of this factor contributes to the ability of microstructured implant surfaces to promote better osseointegration and higher bone-implant contact, since angiogenesis is required for bone formation and maintenance. VEGF-A was also regulated by Dkk1 and Dkk2, since Dkk1 and Dkk2 silenced cells produced lower levels of these factors and their levels were increased by exogenous Dkk2 but not by Dkk1.

The present study shows that Dkk1 and Dkk2 play significant roles in osteoblast commitment, differentiation, and maturation on microstructured surfaces that are in direct contact with bone. We suggest that Dkk1 works by inhibiting the Wnt canonical pathway, while Dkk2 has a dual role, acting as a Wnt canonical inhibitor when LRP5/6-Frizzled complexes are abundant, and as an independent agonist. Inhibition of the Wnt canonical pathway is required in order to activate the BMP-2-Runx2-Osterix-alkaline phosphatase pathway [38] and Dkk1 and Dkk2 appear to play a significant role in this regulation. Our results raise the question of how surface microstructure can modulate Dkk protein expression. Previously, we reported that signaling via the α2β1 integrin is a critical first step in the differentiation of osteoblasts on microtextured Ti surfaces [39–41]. One consequence of this signaling is the production of TGF-β1 and its downstream regulation of osteoprotegerin [42]. Preliminary results indicate that knockdown of the α2 integrin subunit in MG63 cells also reduces Dkk2 expression (Supplemental Fig. 7), suggesting that changes in Dkk2 may mediate the effects of α2β1 signaling on these cells.

5. Conclusion

The results of this study show that production of two Wnt signaling modulators, Dkk1 and Dkk2, are produced by human osteoblasts and MSCs in a surface dependent manner. Levels of mRNA and protein for both are increased in osteoblasts and MSCs grown on TI substrates with micron scale roughness compared to TCPS or smooth surfaces and are

further increased when the microstructured substrate is also hydrophilic. Experiments using MG63 cell lines that have been stably silenced for Dkk1 or Dkk2 show that the two inhibitors act on osteoblasts in an autocrine manner. This was confirmed by treating wild type MG63 cells with antibodies to Dkk2. Effects of Dkk1 are seen in less mature osteoblasts whereas effects of Dkk2 are seen in more mature osteoblasts. Effects of Dkk1 and Dkk2 on MSCs differed from their effects on osteoblasts. These observations indicate that the effects of the two Wnt inhibitors vary with maturation state within the osteoblast lineage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This Study was supported by a grant from the ITI Foundation, NIH AR052102, Children's Healthcare of Atlanta, and the PROFIP of the Universidad Nacional Autonoma de Mexico. Ti disks were provided by Institut Straumann AG (Basel, Switzerland) as a gift. Dr. Marco Wieland was the Vice President for Research at Institut Straumann AG at the time the study was performed; he participated in the study as a contributing scientist.

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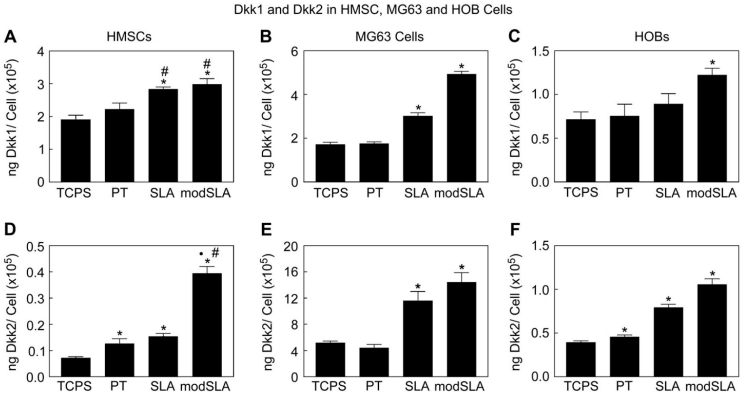

Fig. 1.

Levels of Dkk1 and Dkk2 in conditioned media of cells grown on microstructured titanium surfaces. Dkk1 and Dkk2 were measured in the conditioned media by ELISA of cells grown on tissue culture polystyrene (TCPS) or one of three titanium substrates: smooth PT, microstructured SLA, or SLA with high surface energy (modSLA). Dkk1 and Dkk2 were measured in MSCs (A, D), MG63 cells (B, E) and HOB cells (C, F), respectively. Both Dkk1 and Dkk2 increase in all cell types examined with increasing surface roughness and surface energy.

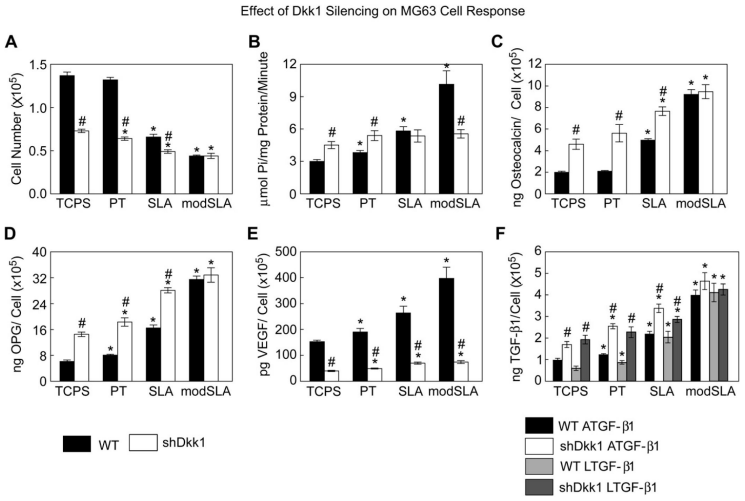

Fig. 2.

Effect of silencing Dkk1 on MG63 cell response to surface microstructure and surface energy. Silencing Dkk1 decreases MG63 cell number and increases osteoblast differentiation and local factor production on TCPS and PT surfaces, but not on the microstructured SLA or modSLA surfaces. MG63 wild type or Dkk1-silenced MG63 cells were cultured on TCPS, PT, SLA, or modSLA surfaces. At confluence, cell number (A) and cellular alkaline phosphatase specific activity (B), as well as the content of osteocalcin (C), osteoprotegerin (OPG) (D), VEGF-A (E) and active and latent TGF-β1 (F) were determined. *$p \leq 0.05$, Ti v. TCPS; #$p \leq 0.05$, wild type v. shDkk1-silenced MG63 cells.

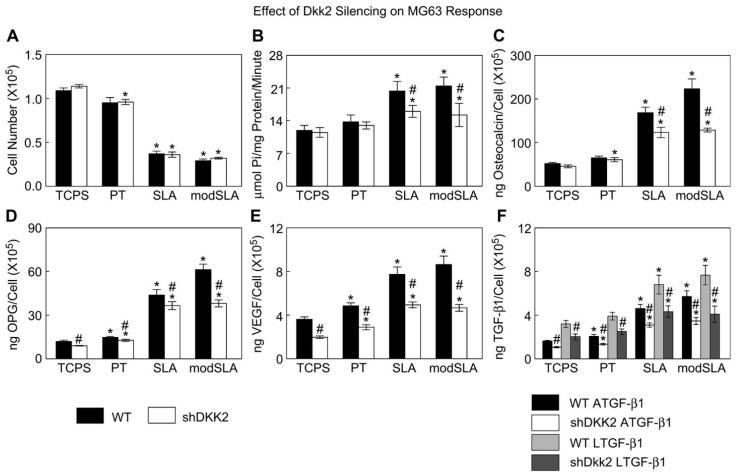

Fig. 3.

Effect of Dkk2-silencing on MG63 cell response to Ti surface microstructure and surface energy. Silencing Dkk2 increases MG63 cell number and decreases osteoblast differentiation and local factor production on SLA and modSLA surfaces, but not on the TCPS and PT surfaces. Cell number (A), and cellular alkaline phosphatase specific activity (B), as well as osteocalcin (C), OPG (D), VEGF-A (E), and active and latent TGF-β1 (F) in the conditioned media were measured. *$p \leq 0.05$, Ti v. TCPS; #$p \leq 0.05$, wild type v. shDkk2-silenced MG63 cells.

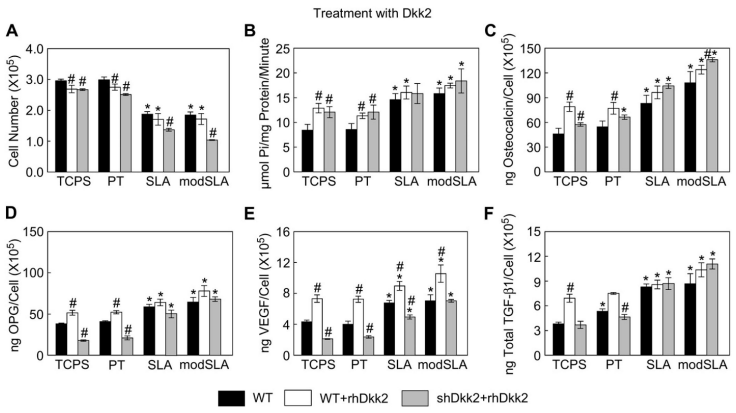

Fig. 4.

Effect of exogenous Dkk1 on MG63 and Dkk1-silenced cell response to surface microstructure and surface energy. MG63 wild type and Dkk1-silenced MG63 cells were cultured on TCPS, PT, SLA, or modSLA surfaces and 1ug/ml rhDkk1 was added to the cultures daily. At confluence, cell number (A) and cellular alkaline phosphatase specific activity (B), as well as the content of osteocalcin (C), osteoprotegerin (OPG) (D), VEGF-A (E) and active and latent TGF-β1 (F) were determined. *$p \leq 0.05$, Ti v. TCPS; #$p \leq 0.05$, treatment with Dkk1 v. untreated MG63 on each surface; •$p \leq 0.05$, Dkk1-silenced MG63 treated with Dkk1 v. wild type MG63 treated with Dkk1.

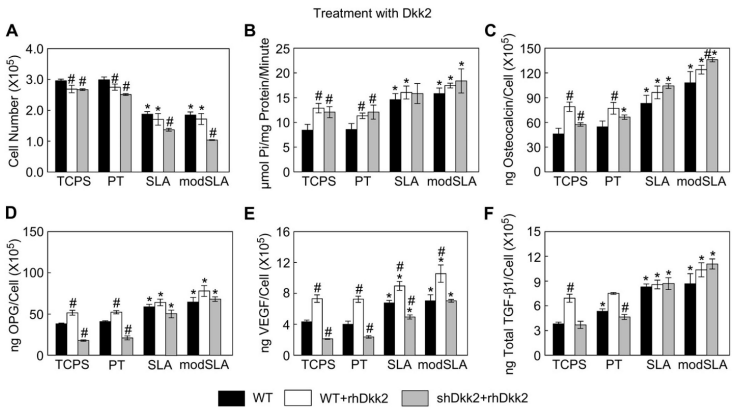

Fig. 5.

Effect of exogenous Dkk2 on MG63 and Dkk2-silenced cell response to surface microstructure and surface energy. MG63 wild type cells and Dkk2-silenced MG63 cells were cultured on TCPS, PT, SLA, or modSLA surfaces and 1ug/ml rhDkk2 was added to the cultures daily. At confluence, cell number (A) and cellular alkaline phosphatase specific activity (B), as well as the content of osteocalcin (C), osteoprotegerin (OPG) (D), VEGF-A (E) and total TGF-β1 (F) were determined. *$p \leq 0.05$, Ti v. TCPS; #$p \leq 0.05$, treatment with Dkk2 v. untreated MG63 on each surface.

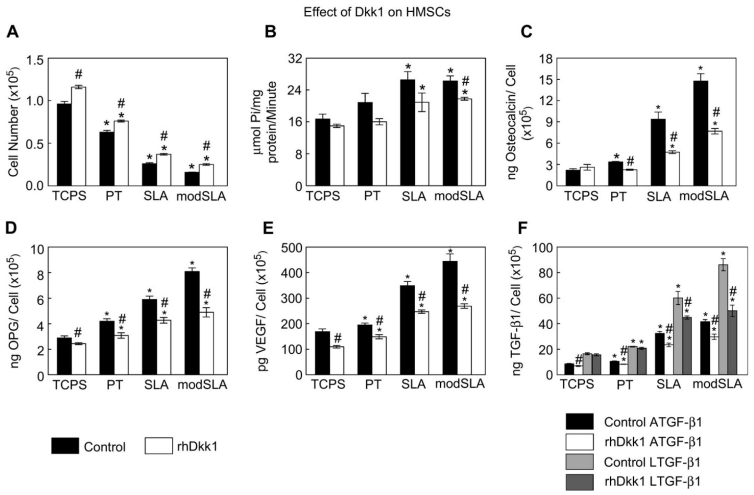

Fig. 6.

Effect of exogenous Dkk1 on MSC response to surface microstructure and surface energy. MSCs plated on TCPS, PT, SLA, or modSLA and were treated with 1ug/mL of rhDkk1 daily until cells reached confluence on TCPS. At confluence, cell number (A), alkaline phosphatase specific activity (B), osteocalcin (C), osteoprotegerin (D), VEGF-A (E) and active and latent TGF-β1 (F) were measured. *$p \leq 0.05$, Ti v. TCPS; #$p \leq 0.05$, rhDkk1-treated MSCs v. MSCs.

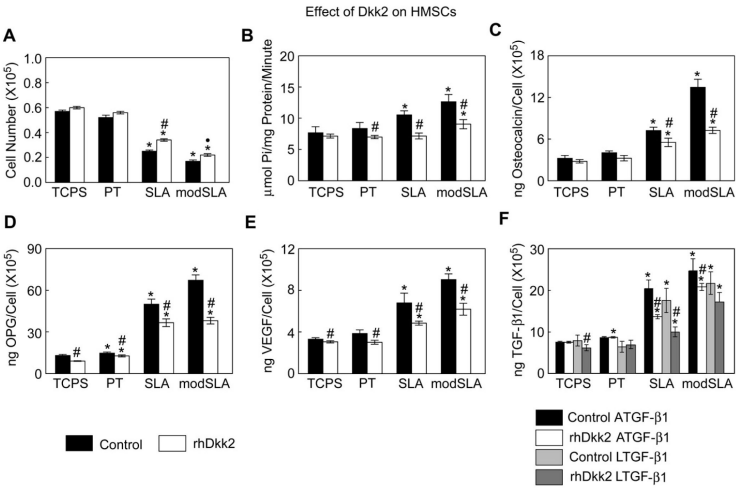

Fig. 7.

Effect of exogenous Dkk2 on MSC response to surface microstructure and surface energy. MSCs plated on TCPS, PT, SLA, or modSLA and were treated with 1ug/mL of rhDkk2 daily until cells reached confluence on TCPS. At confluence, cell number (A), alkaline phosphatase specific activity (B), osteocalcin (C), osteoprotegerin (D), VEGF-A (E) and TGF-β1 (F) were measured. *$p \leq 0.05$, Ti v. TCPS; #$p \leq 0.05$, MSCs v. rhDkk2-treated MSCs.