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THE IMPORTANCE OF WNT PATHWAYS FOR BONE METABOLISM AND THEIR REGULATION BY IMPLANT TOPOGRAPHY

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Abstract

Endosseous implants are important tools to replace missing teeth or damaged tissue segments. Their clinical success depends on their integration in bone and, thus, on the response of bone cells to material and surface characteristics. Recent evidence has shown that surface topography and chemistry affect WNT signalling, a pivotal pathway for the commitment of mesenchymal progenitors to the osteoblast lineage and for bone homeostasis. WNT signalling comprises several cascades that, acting through different effectors, modulate several aspects of cell behaviour.

It has been shown that cells growing on rough titanium surfaces display a different expression profile for WNT factors, and that surface features can alter the response of bone cells to WNT factors. Although the underlying mechanisms to this regulation are still poorly understood, the present review reports intriguing evidence that that cell cytoskeletal signalling is involved in activating WNT signalling in cells growing on rough implant surfaces.

Keywords: Topography; WNT; titanium implants; osteoblasts; cytoskeleton.

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Introduction

Titanium endosseous implants are a versatile tool in medicine, with broad applications both in dentistry, where they are commonly used to support dental prosthesis, and orthopaedics, to replace damaged bone segments. The proper clinical function of an implant requires the formation of sound bone in close contact with the implant body, a process that is generally referred to as osseointegration (Brånemark, 1983; van Steenberghe *et al.*, 1991). Mesenchymal cells and osteoblasts in particular, are the main players in this process, because they deposit the newly formed bone that will encase the implant. Their initial attachment, adhesion, and their response to implants are therefore critical determinants to the subsequent tissue reaction (Anselme *et al.,* 2010; Anselme, 2011). All these events can be affected by the physical and chemical features of the substrate, such as its chemical composition, wettability, and topography, which can, in turn, affect the pattern of protein adsorption on the biomaterial surface. Several authors have shown that surface characteristics can alter cell growth and differentiation, the expression of extracellular matrix components and their mineralisation both *in vitro* (Keller *et al.*, 2003; Schneider *et al.*, 2003; Lossdorfer *et al.*, 2004; Masaki *et al.*, 2005; Arcelli *et al.*, 2007; Wall *et al.*, 2009; Liu *et al.,* 2010; Wennerberg and Albrektsson, 2010; Donos *et al.*, 2011a; Liu *et al.*, 2012) and *in vivo* (Buser *et al.*, 2004; Germanier *et al.*, 2006; Gahlert *et al.*, 2007; Bornstein *et al.*, 2008; Coelho *et al.,* 2011; Donos *et al.,* 2011b; Ivanovski *et al.,* 2011; Park, 2011; Kämmerer *et al.,* 2012). The optimal implant characteristics to ameliorate their integration in bone, including roughness and texture, are however still the object of intense debate. To this purpose, a deeper understanding of the molecular pathways involved in osteoblast differentiation may prove decisive. Recently, the role of WNT signalling in bone formation has been elucidated, and it appears as a fundamental signalling cascade for osteoblast differentiation and bone formation. Recent evidence suggests that these complex pathways may also play a role in surface-induced osteoblast differentiation, and they could represent useful therapeutic targets to improve the clinical performance of endosseous implants. The aim of this review is therefore to examine the available evidence about the role of WNT signalling in bone formation and its regulation by implant surfaces.

WNT signalling

WNT signalling is activated by specific secreted 350-400 amino acid long lipid-modified proteins (Cadigan and

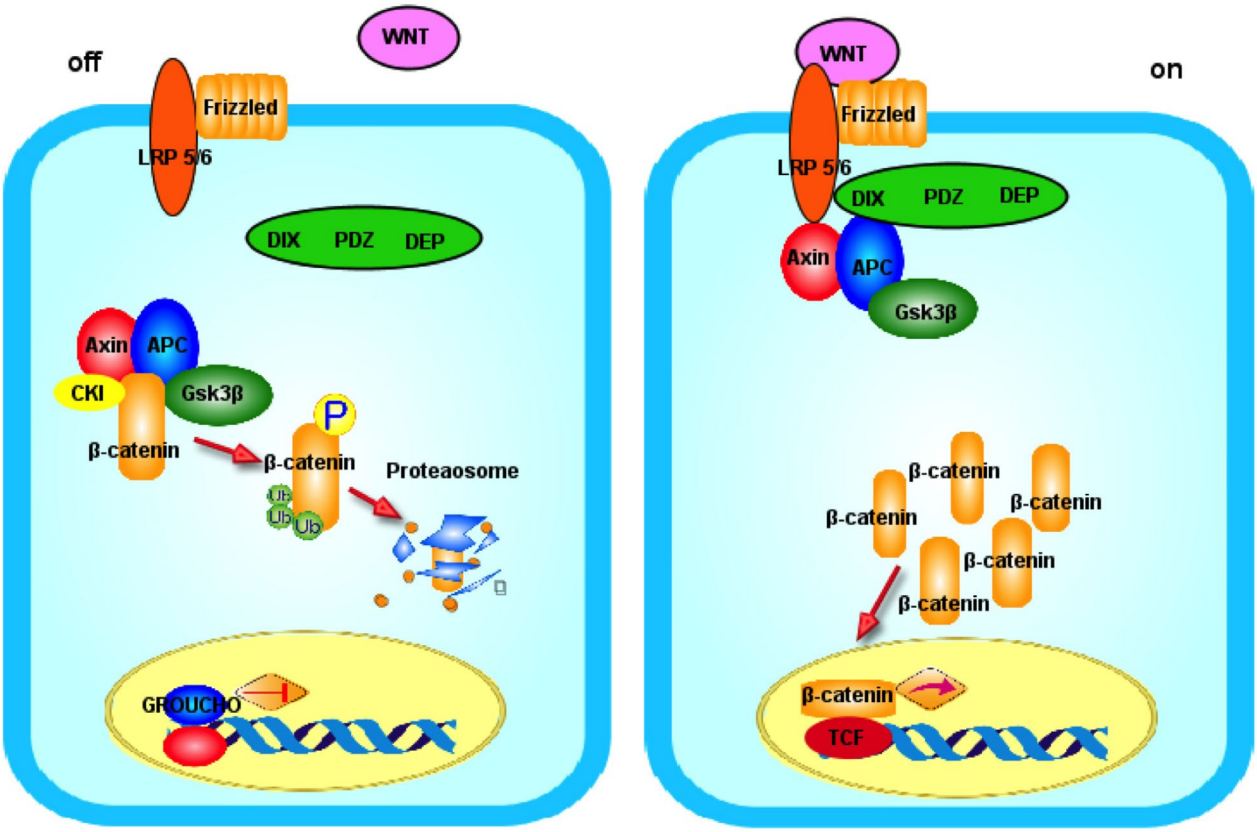

Fig. 1. Diagram showing the main molecular events during WNT canonical signalling. Cytoskeletal β-catenin is phosphorylated by a multimolecular complex and undergoes Ubiquitin (Ub) mediated degradation. Upon binding of specific WNT factors to a Frizzled-LRP receptor complex, the destruction complex is inhibited, thus allowing β-catenin to translocate to the nucleus and bind TCF/Lef.

Nusse, 1997; Katoh and Katoh, 2005). They are known to bind to a receptor complex comprising the Frizzled (Fzd) receptor (van Amerongen *et al.*, 2008; Angers and Moon, 2009) and co-receptors that determine the activation of specific effectors (Mikels and Nusse, 2006). This results in the activation of several different molecular pathways: the WNT canonical pathway, which requires β-catenin (Fig. 1), and a number of so-called non-canonical WNT pathways, among which the Planar Cell Polarity (PCP) pathway and the Ca^{2+}-dependent pathway are the best known (Fig. 2A,B).

WNT canonical pathway

The canonical or WNT/β-catenin pathway requires binding of members of the WNT1 class (WNT1, WNT3, WNT3a, WNT7a, WNT7b, WNT8a) to Fzd and a member of the low-density lipoprotein receptor related protein family, LRP5/6 (Mikels and Nusse, 2006; van Amerongen and Nusse, 2009). This recruits Disheveled (Dvl) to the cell membrane and activates it (Lee *et al.*, 2008; Turm *et al.,* 2010). Dvl consists of three molecular domains: the N-terminal DIX domain, a central PDZ domain, and a C-terminal DEP domain (Axelrod *et al.*, 1998; Boutros and Mlodzik, 1999; Wharton, 2003; Pan *et al.*, 2004). Dvl is considered to play a pivotal role in regulating WNT-activated signalling cascades. It has been shown that the DIX and PDZ domains trigger β-catenin stabilisation (Kishida *et al.*, 1999), while the PDZ and DEP domains are required for activation of WNT non-canonical cascades. In the absence of WNT stimulation, β-catenin is retained in the cytoplasm by a multi-protein degradation complex comprising Glycogen synthase kinase 3 (GSK3) (Force and Woodgett, 2009) and Casein kinase 1a (CKI) (Bernatik *et al.*, 2011), and two scaffold proteins, Adenomatous polyposis coli (APC) (Tanneberger *et al.*, 2011) and Axin (Chia and Costantini, 2005). This leads to β-catenin phosphorylation and its subsequent targeting for proteosomal degradation (Clevers, 2006; Verheyen and Gottardi, 2010).

β-catenin is a multirole protein that can act both as a normal constituent of cell-to-cell junctions by associating to membrane cadherins and as a co-transcription factor. It contains a central domain of 12 Armadillo repeats, which mediate most of the interactions of β-catenin with other proteins (Xu and Kimelman, 2007; Angers and Moon, 2009). Upon receptor activation, Frz binds to Dvl and the PPPS/TP motifs of LRP are phosphorylated, thus recruiting Axin and inhibiting GSK3 activity and the formation of the destruction complex. Recent evidence has demonstrated that LRPs can also be phosphorylated through an alternative, MAPK-dependent mechanism (Cervenka *et al.,* 2011). These events cause the release of β-catenin, which then can translocate to the nucleus, where it interacts with a member of the T cell factor/lymphoid

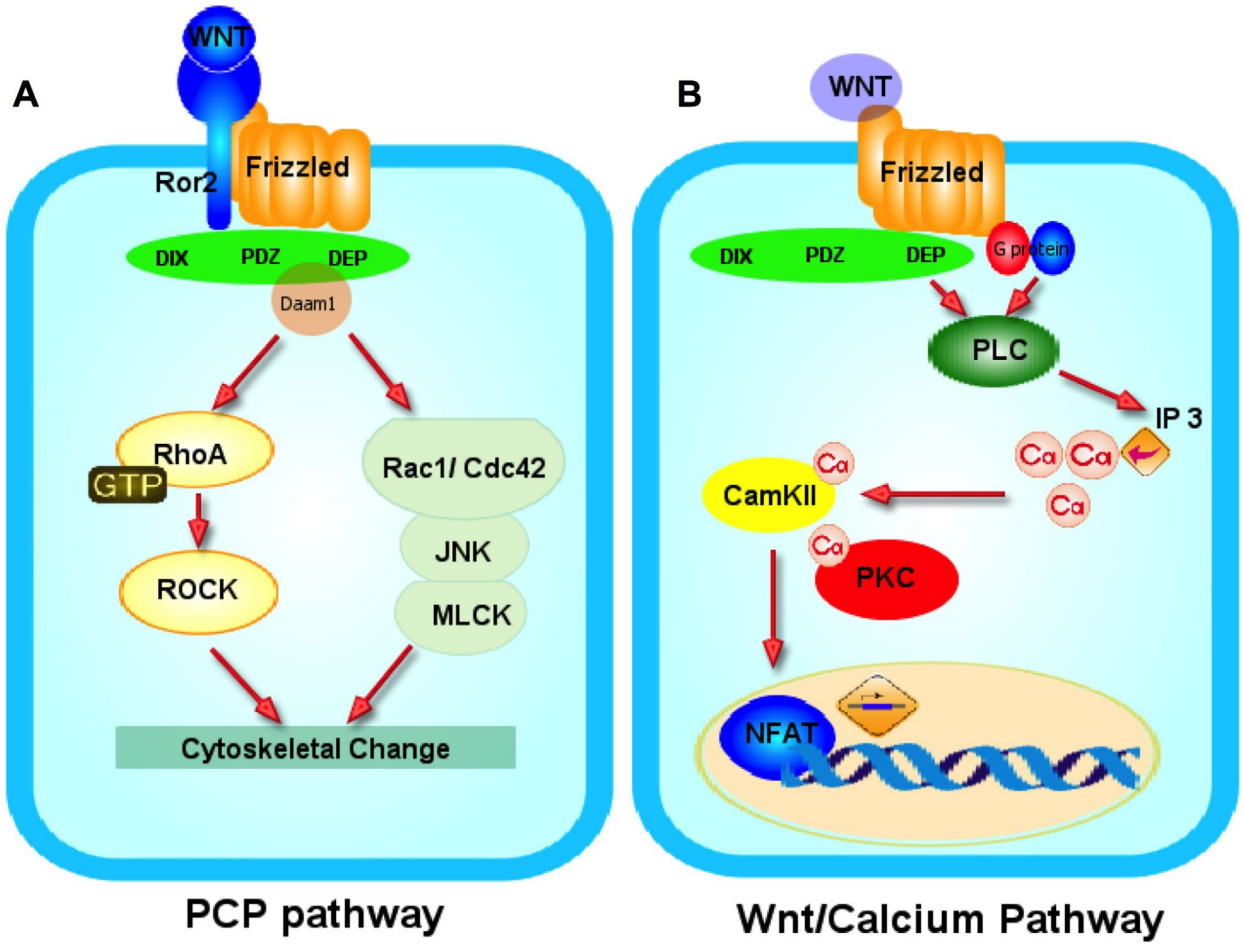

Fig. 2. Diagram summarising the non canonical Planar Cell Polarity (*left panel*) and Calcium-dependent (*right panel*) WNT signalling pathways. Both pathways activate Dishevelled (Dvl) but do not require β-catenin involvement, relying on direct activation of effectors controlling cytoskeletal rearrangement (PCP pathway) or Ca^{2+}-Calmodulin activation.

enhancer factor (TCF/Lef1) transcription factor family (Mosimann *et al.*, 2009), of which several members have been identified (Arce *et al.*, 2006; Hoeppner *et al.*, 2009). The binding of the first 50 amino acids of TCFs and the 3-10 Armadillo repeats of β-catenin dramatically increases TCF/Lef1 affinity for DNA and induces the recruitment of other transcriptional co-activators, such as p300 and cAMP response element-binding protein (p300/CBP), which synergistically regulate the expression of canonical WNT target genes (Tutter *et al.*, 2001; Mosimann *et al.*, 2009).

WNT non-canonical pathways

Some Wnt factors can activate downstream signalling independently of β-catenin, which are collectively known as non-canonical pathways. Two signalling pathways have best been characterised and are denominated Planar Cell Polarity (PCP) pathway and Ca^{2+}-dependent pathway. We will focus our attention on these pathways as they are relevant for the rest of the present review.

PCP pathway

The non-canonical WNT pathway, also known as Planar Cell Polarity (PCP) pathway because of its role in determining tissue organisation, is a β-catenin-independent molecular cascade which is triggered by binding of WNT factors, such as WNT5a, to a Fzd/orphan tyrosine kinase Ror2 receptor complex (Mikels and Nusse, 2006) (Fig. 2A). The PCP pathway activates downstream effector molecules small GTPases RhoA, Rac1 and Cdc42, which have several roles in cell physiology (Schlessinger *et al.*, 2009), through Dvl.

Dvl interacts with Daam1 through its PDZ and DEP domains (Gao and Chen, 2010), and they together interact with the Rho guanine nucleotide exchange factor (GEF), thus inducing the formation of a RhoA-GTP complex, which, in turn, activates ROCK kinase. This pathway has been shown to control actomyosin-induced tensional forces within the cytoskeleton and thus the reorganisation and rearrangement of the cytoskeleton (Gao and Chen, 2010).

Dvl can also activate other small GTPases of the Rho family, namely Rac1 and Cdc42. These GTPases stimulate c-JunN-terminal kinase (JNK) and thus control Myosin Light Chain Kinase and promote actin polymerisation, a feature that is required to generate protrusive forces during cell migration (Jaffe and Hall, 2005; Schlessinger *et al.*, 2009).

In the 1980s, Ingber introduced the tensegrity concept to cell biology (Ingber, 1993). According to this model, the cytoskeleton can be envisaged as a pre-stressed network where microfilaments and intermediate filaments

generate tensional forces that are compensated by stiff microtubules, whose molecular structure allows them to effectively withstand compression, creating a balanced structure under a constant tension (Stamenovic and Coughlin, 1999; Stamenovic *et al.*, 2002; Wang *et al.*, 2002; Ingber, 2003). These principles, originally developed to engineer architectural structures, can effectively prove cell mechanical behaviour. Mechanical stimulation applied to cell membranes can be transferred through the cytoskeleton across the cytoplasm only because its components are under pre-stress (Wang *et al.*, 1993; Wang and Ingber, 1994; Wang *et al.*, 2009b). Pre-stress enables cells to respond to alterations in external forces and changes in the internal pre-stress level may increase or decrease cell sensitivity to mechanical stimuli. Indeed, it has long been known that the shape of a cell can determine its fate (Chen *et al.*, 1997; Smith *et al.*, 2003; Hu and Wang, 2006; Kilian *et al.*; Wozniak and Chen, 2009; Li *et al.,* 2011). Consistently with these ideas, it has been demonstrated that the isometric tension generated by the activation of RhoA and ROCK is required for osteoblast differentiation (Arnsdorf *et al.*, 2009a; Arnsdorf *et al.*, 2009b). The interplay between cell shape and cell fate is only beginning to be elucidated but it is possible that it will play a major role in future research, in particular for its far-reaching implications in cell responses to surface topography (Habas *et al.*, 2003; Milat and Ng, 2009; Rossol-Allison *et al.*, 2009). More will be said about this topic further below.

WNT/calcium pathway

Early studies showed that WNT-5 or Rat Frizzled-2 (RFz-2) overexpression, but not WNT-8 or RFz-1, were able to increase the release frequency of intracellular Ca^{2+} in zebrafish embryos and activate Protein Kinase C (PKC) in Xenopus (Sheldahl *et al.*, 2003; Freisinger *et al.*, 2008). This evidence allowed the identification of a novel non canonical WNT pathway, mediated by calcium release, which was then called WNT-Ca^{2+}-dependent pathway (Fig. 2B).

WNT-Calcium dependent signalling is triggered in response to activation of RFzd-2 receptor or in the presence of WNT-5a/WNT-11 stimulation, without affecting β-catenin stabilisation. The result is the activation of a wide array of effectors including phospholipase C (PLC), and Ca-sensitive enzymes such as Ca-calmodulin-dependent Protein Kinase II (CamKII) and PKC or calcineurin (Kuhl *et al.*, 2000a; Kuhl *et al.*, 2000b).

The activation of PLC by a G-protein β/γ dimer mobilises IP_3 from the cell membrane. This mediates the release of calcium ions from the endoplasmic reticulum and their intracellular accumulation, which activates Ca^{2+}-sensitive proteins PKC and CamKII.

WNT/Frizzled-induced calcium release requires Dvl (Kuhl, 2004). Its PDZ and DEP domains are also capable of stimulating the WNT–Ca^{2+}-pathway in a G protein-independent manner. This suggests that Dvl is either downstream of G protein or acting in parallel with it (Sheldahl *et al.*, 2003).

The role of Wnt signalling in Embryo Development

Several authors have demonstrated that the canonical WNT pathway is required in various events of embryo development such as early trophoblast lineage development, blastocyst activation, implantation, chorionallantois fusion, and embryo morphogenesis (Huelsken *et al.*, 2000; Martin and Kimelman, 2009, Sonderegger *et al.*, 2010). Recent evidence has also shown that Wnt pathways are also involved in the control of embryonic stem cell (ES) proliferation and differentiation (Nusse, 2008; Miki *et al.,* 2011; Sokol, 2011), possibly by modulating Oct4, Nanog, and Sox2, which are known to modulate pluripotency (Sato *et al.*, 2004, Anton *et al.,* 2007). Supplementation with Wnt3a has been shown to maintain murine ES (mES) cell pluripotency (Hao *et al.,* 2006; Singla *et al.,* 2006). Stabilisation of β-catenin through GSK3β inhibitors or GSK3b ablation promoted ES cell self-renewal and expression of stemness-related genes Oct4 and Nanog (Aubert *et al.,* 2002; Sato *et al.*, 2004; Doble *et al.*, 2007). Although Wnt1 overexpression has been reported to attenuate neural differentiation of mES cells (Aubert *et al.,* 2002), some authors reported that Wnt3a stimulated not only human ES cell proliferation, but also differentiation. (Dravid *et al.,* 2005) and conflicting results on the maintenance of stemness and self-renewal by β-catenin/TCF have been published (Pereira *et al.,* 2006; Takao *et al*., 2007; Lyashenko *et al.,* 2011).

The control of bone metabolism by WNT/β-catenin signalling

Beside its role in ES cell physiology, the WNT β-catenin-mediated signalling is a potent player in bone and cartilage metabolism (Krishnan *et al.*, 2006; Chun *et al.,* 2008; Williams *et al.,* 2009). Conditional ablation of β-catenin from osteochondral progenitors in mice resulted in delayed fracture healing (Huang *et al.,* 2012). Expression of a Col2a1-regulated transgene encoding for the β-catenin and TCF inhibitor ICAT, an 82-amino-acid peptide that binds to the armadillo repeats of β-catenin and prevents it from binding TCF, delayed formation of secondary ossification centres, and reduced skeletal growth (Chen *et al.*, 2008). Similarly, Osx1-Cre-mediated conditional deletion of β-catenin in mice disrupted osteoblast differentiation, proving that β-catenin-mediated transcription is necessary for the commitment of osteochondral progenitors to the osteoblastic lineage and their further differentiation into Osterix-expressing pre-osteoblasts (Rodda and McMahon, 2006). Alterations in bone mass have been observed after LRP5 deletion in rodents or as a consequence of mutations of this gene in humans (Gong *et al.*, 2001; Boyden *et al.*, 2002; Kato *et al.,* 2002; Babij *et al.,* 2003; Holmen *et al.,* 2004; Kokubu *et al.,* 2004; Sawakami *et al.,* 2006; Joeng *et al.,* 2011). Since β-catenin is also a membrane component, it is not surprising that the transmembrane protein N-cadherin, a component of cell-to-cell junctions, can act as an inhibitor of WNT/β-catenin signalling by binding and sequestering LRP5 and axin and thus

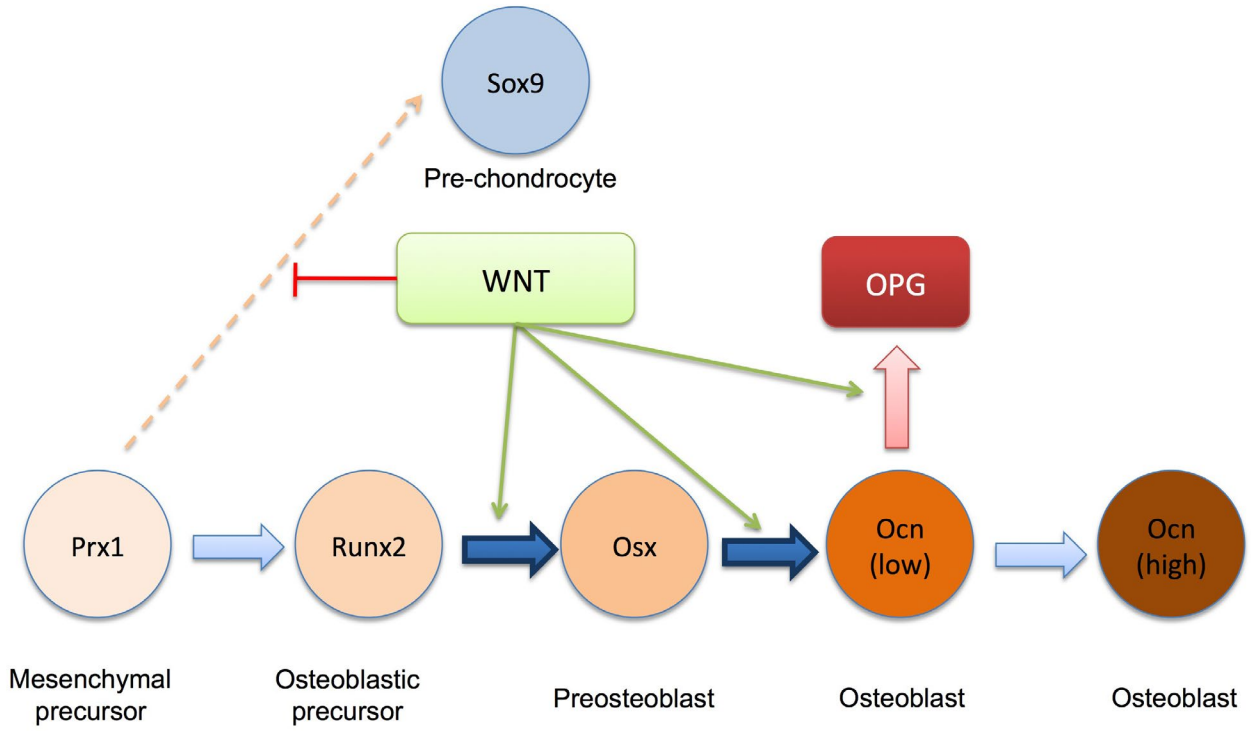

Fig. 3. Scheme showing WNT signalling effects on osteoblastic differentiation. The expression of specific promoters Prx1, Runx2, Osterix (Osx), Osteocalcin (OCN) at low or at high levels is used to label progressive stages of differentiation along the lineage. Canonical, and in part non-canonical, WNT pathways promote commitment to the osteoblastic lineage and expression of osteoprotegerin (OPG), while inhibiting the progression of osteochondral precursors to the chondrogenic fate (modified from Rodda and McMahon, 2006).

reducing bone mass in mice (Hay *et al.*, 2009). Taken together, the available evidence in the literature indicates that β-catenin can act as a molecular switch determining the differentiation lineage that mesenchymal precursors commit to, promoting osteoblast differentiation, and thus bone formation, over alternative cell fates, such as chondrocytes or adipocytes (Hill *et al.,* 2005; Kennel and McDougal, 2005; Rodda and McMahon, 2006) (Fig. 3). Interestingly, inhibition of β-catenin by Chibby (Cby), an evolutionarily-conserved 14.5 kD competitive antagonist (Takemaru *et al.*, 2003) or block of canonical signalling by non-canonical Wnt5b factor is necessary for adipocytic differentiation (Li *et al.*, 2007; van Tienen *et al.,* 2009). Conversely, β-catenin stabilisation has been proven to suppress adipocyte differentiation (Cho *et al.,* 2008). Moreover, β-catenin has been proven to act on mature bone and regulate bone remodelling by inducing osteoprotegerin (OPG) expression (Glass *et al.*, 2005), the decoy receptor of Receptor Activator for Nuclear Factor kB Ligand (RANKL), which is required for osteoclast differentiation. Non-canonical signalling pathways are likely to play an important role too, as recently demonstrated by Chang *et al*. who showed that overexpression of WNT-4 activates a β-catenin-independent signalling cascade and induce osteoblastic differentiation of periodontal ligament-derived MSCs, thus enhancing bone regeneration in periodontal and calvaria bone defect models (Chang *et al.*, 2007). Although ablation studies have repeatedly shown that β-catenin-mediated signalling is required for osteoblastogenesis and bone formation (Hill *et al.,* 2005; Rodda and McMahon, 2006), activation of the canonical Wnt pathway by β-catenin overexpression has yielded conflicting results on both osteoblast differentiation *in vitro* (Fukuda *et al.,* 2010) and bone formation *in vivo* (Hill *et al.,* 2005), suggesting that a more complex modulation of the signal and activation of parallel pathways are required for correct bone accrual.

The effects of titanium topography on WNT signalling pathway

The topography of titanium surfaces influences the biological responses of cells, of surrounding tissues, hence affecting implant osseointegration (Boyan *et al*., 2002; Linez-Bataillon *et al*., 2002; Ramires *et al*., 2002; Buser *et al*., 2004; Lossdorfer *et al*., 2004; Schneider *et al*., 2004; Isa *et al*., 2006; Germanier *et al*., 2006). Although this phenomenon has long been known, the molecular mechanisms responsible for the effect of surface roughness on cell function are still poorly understood.

It is known that osteoblasts growing on microstructured Ti surfaces produce local factors that regulate bone formation as well as bone remodelling, including the expression of RANK ligand decoy receptor osteoprotegerin (OPG), under the control of a2β1 integrins (Schwartz *et al.*, 2009). Even nanoscale topographies can alter cell gene expression (Dalby *et al.,* 2008).

The gene expression profile associated with the early response biological processes during the osseointegration of implants with rough surface is characterised by an over-representation of genes associated with proliferation and immuno-inflammatory response, and, at a later stage, of genes associated with skeletogenesis and angiogenesis (Ivanovski *et al.,* 2011). Moreover, in agreement with these findings, the *in vivo* gene expression profile of guided bone regeneration around a micro-rough titanium surface showed a profound up-regulation of skeletogenesis-related genes on sand-blasted and acid etched (SLA) surface, accompanied by an increase in angiogenesis-associated gene expression (Donos *et al.,* 2011b).

In vivo and *in vitro* whole genome analysis (Wall *et al.*, 2009; Donos *et al.,* 2011b; Ivanovski *et al.,* 2011) identified WNT signalling as the predominant signalling pathway differentially regulated by surface features such as topography and wettability, and it is currently deemed the most important pathway in the regulation of cell differentiation in response to implant surfaces. WNT pathways are activated on rough surface topography through over expression of WNT ligands such as WNT2b, WNT8b, WNT10a and of LRP5 receptor, thereby resulting in enhanced cell differentiation on rough SLA surface as confirmed by enhanced expression of osteoblast specific genes alkaline phosphatase (ALP), osteocalcin (OCN), OPG, Runx2 and TGF-β1 (Olivares-Navarrete *et al.,* 2011a).

The involvement of WNT signalling on modulation of cell behaviour on rough titanium surface was also proven by the role of WNT signalling modulators on osteoblast differentiation on microstructured titanium surfaces. A large number of antagonists can modulate WNT signalling. These include the Dickkopf (Dkk) family, WNT inhibitory factor (WIF), soluble Frizzled-related proteins (sFRP), and Cerberus and Sclerostin families. Both Dickkopf-1 (Dkk1) and Dickkopf-2 (Dkk2) inhibit the WNT signalling pathway by binding to LRP5/6 and preventing formation of the WNT- Fzd-LRP complex (Qiang *et al.*, 2008a; Olivares-Navarrete *et al.,* 2010a). Olivares-Navarrete *et al*. recently demonstrated that expression and activity of Dkk1 and Dkk2 was modulated in MSCs, HOBs and MG63 cells on Ti surfaces with rough microstructure (Olivares-Navarrete *et al.,* 2010a). Moreover, Dkk1 silencing increased the expression of differentiation markers OCN and OPG on smooth substrates but not on rough Ti. Silencing Dkk2 reduced stimulatory effects of SLA and modSLA on osteoblast differentiation because it reduced production of autocrine regulators of osteoblasts differentiation (TGFβ1) and osteoclast activity (OPG, TGFβ1). Dkk1 and 2 also prevented MSC differentiation, thus promoting an undifferentiated stem cell phenotype (Olivares-Navarrete *et al.,* 2010a).

Data from our group showed that more β-catenin translocated to the nucleus in cells on SLA surfaces after stimulation with WNT3a and stimulation of the β-catenin/TCF signalling was accountable for at least part of the effects of surface on cell differentiation (Galli *et al.*, 2010). Rough surfaces enhanced the WNT3a-induced expression of β-catenin target genes ALP, OPG, Wisp2, Connexin 43 (Cx43) and Cyclooxygenase 2 (Cox-2). The induction of a β-catenin mutant form (S33Y) that could not be phosphorylated by the destruction complex and thus be degraded reversed the effects of surface topography on β-catenin-mediated transcription, suggesting that surface topography may have a permissive effect on WNT canonical signalling by acting on β-catenin degradation (Galli *et al.*, 2010).

Interestingly, surface topography can affect other β-catenin-dependent molecular cascades, such as the FoxO pathway, which is responsible for cell defences against oxidative stress. We showed that FoxO/β-catenin signalling was elevated in cells on rough surfaces, as demonstrated by increased FoxO-Luc activity and higher mRNA levels for FoxO-target genes MnSOD, Catalase, GADD45 (Galli *et al.,* 2011). Although the molecular basis of the higher activity of FoxO-mediated pathway on rough surfaces is still to be investigated, it is possible that the same mechanisms underlie the activation of these β-catenin-dependent pathways, possibly a higher availability of free cytosolic β-catenin. A reduced sequestering of β-catenin by membrane-associated Cadherins, due to reduced cell-to-cell junctions and increased cell spacing on rough and irregular surfaces, may play a role in this phenomenon.

Moreover, it has been demonstrated that both ALP activity and OCN in MG63 and hMSC cells increased on titanium surfaces in the presence of exogenous non canonical WNT5a, reaching highest levels on rough surfaces, whereas WNT3a treatment failed to affect osteoblastic gene expression (Olivares-Navarrete *et al.,* 2011a; Olivares-Navarrete *et al.,* 2011b). Indeed, whole-genome analysis revealed that the osteogenic mediator WNT5a was up-regulated on rough titanium (Wall *et al.*, 2009). This would support the idea that non-canonical WNT pathways may play an important role in differentiation of cells on microstructured titanium surfaces alongside with the canonical cascade. Although it is not clear which non-canonical pathways are modulated by surface topography, the PCP pathway seems to be involved in the regulation of cell orientation on microstructured implant surfaces (Calzado-Martin *et al.,* 2011). Moreover, Wnt5a upregulation may indicate that Ca-dependent cascade is activated on rough surfaces, although this factor has been shown to can exert its action through alternative cascades such as by activating RhoA in mechanical stimulation of osteoblasts (Arnsdorf *et al.,* 2009a; Arnsdorf *et al.,* 2009b). It is important to remember that even well-established canonical factors such as Wnt3a can activate parallel signalling pathways that have been shown to affect bone formation (Tu *et al.,* 2007).

In recent years, in addition to topographical changes, chemical modification of titanium has become a focus of attempts to positively influence the process of osseointegration.

Hyperhydrophilic titanium surfaces surfaces (modSLA), obtained by rinsing under N_2 protection and storage in an isotonic NaCl solution (Rupp *et al.*, 2006), maintain the topography of the original surface, but exhibit increased

surface free energy and lower water contact angles (0° compared to 139.98° for conventional SLA surfaces) (Schwarz *et al.*, 2007; Olivares-Navarrete *et al.*, 2010b).

Higher surface energy of titanium surfaces has been shown to enhance cell adhesion in the early stages of cell response and increase the expression of Focal Adhesion Kinase (Lai *et al.,* 2010,). Donos and colleagues, using the genome-wide analysis, studied the gene expression profile of osseointegration associated with a moderately rough (SLA) and a hydrophilic/moderately rough surface (modSLA) in a human model. They inserted titanium endosseous implants with different topography in the retromolar area of volunteer subjects and observed up-regulation of osteogenesis- and angiogenesis-associated gene expression on modSLA surfaces (Donos *et al.,* 2011a) over a time of up to 2 weeks. Several other genes relevant to bone maturation, such as OCN, BMP2, CTNNA1, FZD6 and effectors of the TGFβ-BMP signalling cascade were significantly up-regulated on modSLA samples (Vlacic-Zischke *et al.,* 2011).

It is important to point out that, in contrast to the available general literature on cell responses to implant surfaces, a limited array of surface treatments have been used as experimental models for the regulation of Wnt signalling by implant surfaces, and no study has investigated whether the isotropic or anisotropic features of the surface or other geometric characteristics of the implant surface, which have been shown to play a relevant role in affecting cell activity (Bigerelle *et al.*, 2002; Bigerelle *et al.,* 2011) can actually influence this particular pathway.

Topography, cytoskeleton and cell differentiation

The relationship between cell shape and cell fate has long been subject of speculation and research. It is known that cell spreading leads to higher cytoskeletal tension and differential expression of the small GTPase RhoA and its downstream effector Rho-associated protein kinase (ROCK) (McBeath *et al.*, 2004). The activation of Rho and ROCK plays a significant role in regulating cytoskeletal dynamics and generates isometric tension within the cytoskeleton, acting as a switch for osteoblastogenesis (Kanno *et al.,* 2007; Salasznyk *et al.,* 2007; Kearney *et al.,* 2010). Oh and co-workers studied the differentiation of MSCs on titanium oxide nanotubes of different size and found that increased cell spreading on 70 nm nanotubes was associated to increased osteogenesis. It is likely that elongated morphology caused cellular cytoskeletal tension in hMSCs cultured on 70 to 100 nm diameter nanotubes as compared with cells growing on a flat surface (Oh *et al.*, 2009).

The hypothesis that local shape promotes cell contractility and tension, which in turn enhances differentiation along the osteoblastic lineage was confirmed in a recent work by Kilian *et al.*, who observed that cell shapes promoting increased contractility (star-like shapes) led to higher expression of osteoblast specific genes (Kilian *et al.*, 2010). In contrast, cell shapes that required low contractility (flower-like shapes) promoted cell differentiation along the adipocytic lineage. Moreover, treatment with cytoskeleton-disrupting agents decreased cytoskeletal contractility and thus inhibited osteoblastogenesis. Gene expression analysis by DNA microarrays revealed higher levels of transcript for pro-osteogenic WNT-associated factors (including down-stream effectors RhoA and ROCK) in star-shaped cells, highlighting the importance of adhesion and a highly contractile state for bone cell formation (Kilian *et al.,* 2010). Furthermore, it has been reported that matrix mechanical characteristics, i.e. stiffness, affect stem cell and osteoblast differentiation (Engler *et al.,* 2006; Chatterjee *et al.,* 2010; Pek *et al.,* 2010) and that lineage commitment requires myosin-mediated cell contractility (Engler *et al.,* 2006).

Consistently with these findings, RhoA has also been demonstrated to be required to activate PI3K and MAPK signalling in osteoblastic MC3T3 cells under mechanical stimulation (Hamamura *et al.,* 2012), and integrin-mediated mechanical stimulation of Focal Adhesion Kinase (FAK) has long been recognised as necessary for mechanical transduction in osteoblastic cells (Ponik and Pavalko, 2004; Boutahar *et al.,* 2004; Young *et al.,* 2009; Wang *et al.,* 2011; Young *et al.,* 2011). Biggs *et al.* demonstrated that surface topography directly affects formation of adhesion complexes (Biggs *et al.,* 2009), and this could contribute to modulating the mechanical stimulation of cells on implant substrates. Although molecular pathways by which cell shape and cytoskeletal mechanics drive cell commitment are still uncertain, it becomes evident that topology surface cues can be used to enhance and rationally control differentiation specific signalling, thereby accelerating guided differentiation in engineered scaffolds.

Perspectives

Based on the important role of WNT signalling in osteogenesis, it has been proposed that stimulation of WNT pathways may improve implant osseointegration. To this purpose, WNT growth factors seem like attractive candidates to stimulate bone formation.

A recent paper by Popelut *et al*. (Popelut *et al.,* 2010) showed that liposomal WNT3a transiently enhanced the physiological WNT response that occurs after fractures and implant placement. Peri-implant tissues treated with liposomal WNT3a showed a significant up-regulation of Collagen type I and ALP. Moreover, sites treated with liposomal WNT3a exhibited more bone-to-implant contact surface, with mineralised osteoid matrix in close proximity to the implant surface, thus demonstrating that transient exposure to WNT3a induces peri-implant cells to rapidly commit to an osteogenic lineage.

Stimulation of WNT signalling by strontium induces proliferation and differentiation of murine osteoblasts (Fromigue *et al.,* 2010). The authors showed that strontium ranelate (SnRel) increased WNT3a and WNT5a expression as well as β-catenin transcriptional activity in osteoblasts. Yang *et al*. demonstrated that the activation of WNT pathway, which was induced by strontium, not only promoted the *in vitro* differentiation of both murine osteoblasts and human MSCs, but also significantly

enhanced *in vivo* bone formation (Yang *et al.,* 2011). Strontium-containing hydroxyapatite scaffolds enhanced the accumulation of extracellular matrix in the bone defect and enhanced β-catenin expression *in vivo* (Yang *et al.,* 2011). Although preliminary, their important work suggests that this approach may be proposed to promote implant osseointegration.

Lithium chloride was recently shown to activate the canonical WNT signalling pathway, both *in vitro* and *in vivo*. LiCl inhibits glycogen synthase kinase 3β (GSK3β), an enzyme that phosphorylates β-catenin in the cytoplasm targeting it for ubiquitination and degradation. It also activates WNT signalling independently of WNT receptor, resulting in significantly increased bone formation and bone mass in rodents (Clement-Lacroix *et al.*, 2005). Wang and colleagues used the electrolytic deposition of lithium into calcium phosphate coatings and showed that both attachment and early proliferation of MG63 cells on these hybrid coatings were enhanced and that, though lithium incorporation interferes with calcium phosphate deposition, it ameliorates coating biocompatibility (Wang *et al.*, 2009a).

Although much evidence points at WNT stimulation as a viable approach to induce bone formation, some studies represent an important warning that caution must be exerted when modulating this signal pathway. A recent work by Kim *et al*. suggests that up-regulation of WNT canonical signalling alone, though required for bone healing, may not be sufficient for mature bone formation (Kim *et al.*, 2007) and that, albeit necessary for Osterix expression, stabilisation of β-catenin inhibits Sox9 and Runx2 expression, and thus bone formation (Hill *et al.,* 2005). *In vitro* studies showed that activation of the canonical WNT pathway by WNT-1 or WNT-3a can actually suppress osteogenic differentiation in mesenchymal stem cells (MSC) (Boland *et al.*, 2004; De Boer *et al.*, 2004). Interestingly, ablation of any Wnt factor has not duplicated the arrest in osteoblastogenesis observed with LRP5 or β-catenin deletion, suggesting that various Wnts could act redundantly in this process and most likely act in a coordinated fashion, where both timing and expression patterns contribute to lead mesenchymal cells along the osteoblastic lineage (Hill *et al.,* 2005). Consistently with these studies, the WNT competitive antagonist soluble Frizzled Receptor Protein 3 (sFRP3) increased production of the osteoblastic marker osteocalcin in the osteoblastic MC3T3 cell line (Chung *et al.*, 2004). Other inhibitors of WNT canonical signalling, Dkk1 and Dkk2, appeared to be required for full osteoblastic differentiation (Li *et al.*, 2005; van der Horst *et al.*, 2005), although Dkk1 is also known to inhibit osteoblast function in myeloma-induced bone lesions (Qiang *et al.*, 2008b; Heath *et al.*, 2009) and, conversely, WNT3a signalling within bone can inhibit the growth of this tumour (Qiang *et al.*, 2008c). This amount of data shows that WNT proteins exert a very complex modulation of osteoblast differentiation, through temporally distinct expression patterns (Zhang *et al.*, 2008) in different physiological situations, about which little is known and that future studies will have the task to explore in detail.

Conclusions

WNT pathways have been proven to regulate osteoblast commitment and control bone formation and remodelling. Recent literature has shown that implant topographical characteristics or topography can modulate both canonical and non-canonical pathways in mesenchymal cells. Based on the available data, surface control of WNT signalling appears as one of the prominent mechanisms by which implant surfaces can direct cell commitment and differentiation. Control of this signalling pathway appears therefore as a promising approach to improve implant osseointegration and possibly clinical success, and future studies will have to thoroughly explore the molecular mechanisms through which surface topography can affect WNT signalling in cells and how to regulate the activation of this pathway to enhance osteogenesis.

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Discussion with Reviewer

Reviewer I: Our next challenge for improved osseointegration is for those groups of patients where osseointegration has a poorer clinical outcome. Could the authors speculate whether developments of therapeutics for stimulating WNT signalling would have benefits for any of these compromised patients?

Authors: The real benefit with the stimulation of Wnt or other biological pathways is in those situations where success rate with standard surgical techniques would be quite low, such as in grafted areas or in compromised patients, or wherever bone regeneration is to be promoted to compensate for previous bone loss. We feel that Wnt stimulation would be an appropriate approach to promote per-implant alveolar regeneration because, although scientific evidence is still lacking, Wnt regulation is one of the likely candidate mechanisms regulating alveolar bone maintenance. There is solid evidence pointing at the pivotal anabolic role of mechanical stimulation on bone through the suppression of sclerostin and thus stimulation of Wnt signalling. It is well possible that tooth loss heavily affects alveolar bone levels through a local increase of sclerostin due to a lack of mechanical stimulation. Wnt suppression could therefore be one of the causes of alveolar bone resorption with edentulism. If this is the case, restoring an appropriate Wnt stimulation would be a physiological way to increase and maintain a healthy level of Wnt activity, and thus bone, around implants.

Reviewer I: In their review the authors focus on implant topography as a regulator of the Wnt pathway. As a guidance cue, implant topography can/should be considered along with other guidance cues that would likely exist in combination with the topography, such as surface chemical composition and implant stiffness. What are the authors views on any potential hierarchy of such guidance cues, in terms of regulating the Wnt pathway in osteogenic cells?

Authors: The reviewer's question touches upon a very important conundrum, which is hitherto still unresolved. As far as it is known, all these factors can affect the regulation of Wnt. We have shown that topography can upregulate Wnt canonical signalling, a paper from our group, which is currently in press, shows that the activation of Wnt signalling is higher in cells hyperhydrophilic modSLA surfaces, and it can be speculated that, on a tissue level, implant stiffness can affect the transmission of mechanical stimuli to the surrounding bone and indirectly modulate Wnt signalling by altering, for instance, sclerostin expression. However, little evidence is available to reach a solid conclusion about a possible hierarchy of factors. So far we have conclusive data only about signalling regulation by roughness. When it comes to hydrophilicity, we know that it does enhance Wnt canonical signalling, but we do not believe that polished hyperhydrophilic surfaces have ever been tested in this regard so we do not know the net effect of this feature on this pathway. We only know that modSLA surfaces induce a higher activation of Wnt signalling than SLA samples and polished surfaces. Again, when it comes to implant stiffness, it is likely that it can exert some sort of effect on the surrounding tissue but probably not at the cell level, at least in the stiffness range of cpTi or titanium alloys. Softer surfaces, like hydrogels, can affect cell differentiation, but we doubt that cells can discriminate between such high stiffness levels. In conclusion, probably roughness remains the key element, at least until more is known about hydrophilicity.