ORIGINAL RESEARCH

WNT16 Requires Gα Subunits as Intracellular Partners for Both Its Canonical and Non-Canonical WNT Signalling Activity in Osteoblasts

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Abstract

In the past years, WNT16 became an interesting target in the field of skeletal research, as it was identified as an essential regulator of the cortical bone compartment, with the ability to increase both cortical and trabecular bone mass and strength in vivo. Even though there are indications that these advantageous effects are coming from canonical and non-canonical WNT-signalling activity, a clear model of WNT signalling by WNT16 is not yet depicted. We, therefore, investigated the modulation of canonical (WNT/β-catenin) and non-canonical [WNT/calcium, WNT/planar cell polarity (PCP)] signalling in human embryonic kidney (HEK) 293 T and SaOS2 cells. Here, we demonstrated that WNT16 activates all WNT-signalling pathways in osteoblasts, whereas only WNT/calcium signalling was activated in HEK293T cells. In osteoblasts, we therefore, additionally investigated the role of Gα subunits as intracellular partners in WNT16′s mechanism of action by performing knockdown of Gα12, Gα13 and Gαq. These studies point out that the above-mentioned Gα subunits might be involved in the WNT/β-catenin and WNT/calcium-signalling activity by WNT16 in osteoblasts, and for Gα12 in its WNT/PCP-signalling activity, illustrating a novel possible mechanism of interplay between the different WNT-signalling pathways in osteoblasts. Additional studies are needed to demonstrate whether this mechanism is specific for WNT16 signalling or relevant for all other WNT ligands as well. Altogether, we further defined WNT16′s mechanism of action in osteoblasts that might underlie the well-known beneficial effects of WNT16 on skeletal homeostasis. These findings on WNT16 and the activity of specific Gα subunits in osteoblasts could definitely contribute to the development of novel therapeutic approaches for fragility fractures in the future.

Keywords Wnt16 · WNT signalling · Osteoblasts · Gα subunits

Introduction

After decades of research, the Wingless-type mouse mammary tumour virus integration site (WNT)-signalling pathway has become a major topic in the understanding of

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skeletal homeostasis and the development and treatment of skeletal disorders. This WNT-signalling pathway is mostly subdivided in three distinctive WNT pathways; the canonical or β-catenin-dependent (WNT/β-catenin) pathway and two non-canonical or β-catenin independent pathways (WNT/calcium (Ca^{2+}) and WNT/planar cell polarity (PCP) pathway) [1]. These pathways are modulated by WNT proteins that bind to one of the 10 Frizzled (Fzd) receptors, serpentine receptors structurally classified as G protein-coupled receptors (GPCRs) [2]. What differentiates Fzd receptors from other GPCRs, however, is that Fzd receptors can transmit signals by the downstream dishevelled protein or by heterotrimeric G proteins [3]. Upon today, especially the role of these heterotrimeric G proteins in the WNT signalling pathway remains unclear.

As previously mentioned, WNT signalling is considered as an important regulator of skeletal homeostasis. These findings are partially based on the identification of

disease-causing mutations in several players of the WNT pathway in patients with different monogenic bone disorders [4]. In addition, in the past decade, many candidate gene and genome-wide association studies (GWAS) demonstrated that several loci that contain genes which cluster within the WNT-signalling pathway [5, 6] contribute to the development of osteoporosis. Osteoporosis is the most common metabolic bone disorder and is marked by a decreased bone mass and quality which results in an increased fracture risk. Currently, one in three women and one in five men above the age of 50 experience an osteoporotic fracture [7]. This makes osteoporosis a defining health problem of our ageing society, and therefore, encourages the biomedical community to better understand, prevent and cost-effectively treat this skeletal disorder.

In the past 5 years, *WNT16* was pointed out by numerous genetic studies as an essential determinant of BMD at different skeletal sites and of the risk of fragility fractures [5, 8–15]. Functional validation of the relevance of WNT16 in skeletal homeostasis came with the analysis of mice with global or osteoblast-specific knockout (KO) of *Wnt16*. Global and osteoblast-specific conditional KO of *Wnt16* both result in significantly thinner and more porous cortical bones and an increased fracture risk, whereas the trabecular bone remains unaffected [12, 15, 16]. However, transgenic mice overexpressing *Wnt16* in osteoblasts or osteocytes displayed a higher BMD and improved bone strength in both the trabecular and cortical bone compartment [17–19]. Off all skeletal cell types, WNT16 is predominantly expressed in osteoblasts, from where it is known to affect osteoclastogenesis, but not osteoclast function, in a direct and indirect manner [16]. So far, these effects of WNT16 are carefully attributed to activation of the WNT/β-catenin pathway and a non-canonical JNK cascade in osteoblasts, whereas it solely activates non-canonical WNT signalling in osteoclasts [16]. Altogether, these findings highlight WNT16 as a key regulator of cortical bone mass and fragility fractures, but the precise effects of WNT16 on the different WNT-signalling pathways are poorly understood [20].

Based on previous experiments WNT16 appears to be unable to activate WNT/β-catenin signalling in human embryonic kidney (HEK) 293 T cells [10] while it can activate this pathway in MC3T3-E1 pre-osteoblasts [16, 21]. This suggests that WNT16 has an osteoblast-specific mechanism of action, with beneficial influences on trabecular and cortical bone. Although some information on this mechanism of action is already available, a clear model of WNT16 signalling in osteoblasts was not yet depicted. Further elucidation of this mechanism could be of great interest to further explore different anabolic processes and putative therapeutic approaches for fragility fractures in osteoporosis patients.

The aim of this study was thus to depict a model for WNT16 signalling by investigating the modulation of canonical and non-canonical WNT signalling by Wnt16 and look for a possible mechanism explaining the observed effects. Therefore, we performed different luciferase reporter assays in both HEK293T and SaOS2 cells, an osteoblast-like cell line, to examine the modulation of WNT signalling by Wnt16 in both cell types. Finally, we examined whether Gαq, Gα12 and Gα13 subunits could be intracellular partners in Wnt16′s mechanism of action, by additionally performing knockdown experiments in osteoblasts.

Materials and Methods

Cell Culture, Transfection and Luciferase Reporter Assays

The HEK293T and SaOS2 human osteosarcoma cell line (ATCC) were grown in DMEM supplemented with FBS (10% v/v, Life Technologies, Carlsbad, CA, USA). Cells were maintained at 37 °C in 5% CO_2 and 95% air atmosphere. Both HEK293T and SaOS2 cells were passaged when confluent by standard trypsinization with TrypLE™ Express (Life Technologies, Carlsbad, CA, USA) and subculturing procedure. Twenty-four hours prior to transfection, HEK293T or SaOS2 cells were plated at 0.3×10^5 cells/well in 96-well plates for standard luciferase reporter assay experiments. FuGENE 6 and ViaFect Transfection Reagent (Promega Corporation, Madison, WI, USA) were used for transfection of HEK293T and SaOS2 cells, respectively, according to the manufacturer's instructions. In HEK293T cells, different amounts of the pCMV6-Entry vector containing murine Wnt16 cDNA (10–40 ng) were co-transfected with pRL-TK (1 ng) and a pGL4.30 (20 ng, Promega Corporation, Madison, WI, USA) or pGL4.44 (20 ng, Promega Corporation, Madison, WI, USA) luciferase reporter vector with NFAT or AP-1 response elements, respectively. In SaOS2 cells, pRL-TK (2 ng) and pGL3-OT (30 ng) were co-transfected together with different amounts of pCMV6-Entry-Wnt16 (20–40–80 ng) to monitor WNT/β-catenin signalling. Increased expression of Wnt16 in SaOS-2 cells after transfection was confirmed using quantitative reverse transcriptase PCR (qRT-PCR, Supplemental Fig. 1). Co-transfection of different amounts of pCMV6-Entry-Wnt16 with pRL-TK (2 ng) and pGL4.30 (30 ng), pGL4.44 (30 ng) or pCRE-Luc (30 ng, Stratagene, La Jolla, Ca, USA) in SaOS2 cells was performed to monitor WNT/calcium, WNT/PCP or cAMP-CREB signalling, respectively. When needed, empty pcDNA3.1 vector was added to make the total DNA amount equal for all transfection experiments. Each transfection was carried out in triplicate and repeated independently in three separate experiments. Forty-eight hours after transfection, cells were lysed and firefly and Renilla luciferase activity were measured on a Glomax Multi+Luminometer (Turner

Designs, Sunnyvale, CA, USA) using the dual luciferase reporter assay system (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. The ratio of the firefly and Renilla luciferase measurement was calculated. Data are expressed as mean values ± SD.

GNA12, GNA13, GNAQ DsiRNAs and Transfection

For knockdown of *GNA12* (NM_007353), *GNA13* (NM_006572) and *GNAQ* (NM_002072), three TriFECTa RNAi Kits (Integrated DNA Technologies, Coralville, IA, USA) were purchased, each including 3 Dicer-substrate 27-mer duplexes (DsiRNAs) targeting one specific gene (sequences available upon request). Prior to transfection, DsiRNAs were heated to 94 °C for 2 min to have fully resuspended DsiRNAs in a stable and double-stranded form. To obtain optimal transfection conditions, co-transfection of empty pcDNA3.1 vector and a TYE 563-labelled control siRNA duplex (Integrated DNA Technologies, Coralville, IA, USA) in SaOS2 cells was performed and transfection efficiency was verified using fluorescence microscopy. A scrambled (NC1, Integrated DNA Technologies, Coralville, IA, USA) and human hypoxanthine phosphoribosyltransferase (HPRT) siRNA duplex were used as negative and positive controls, respectively, to confirm transient transfection and knockdown efficiency. For efficient knockdown of the selected Gα subunits, 20 nM of one TriFECTa siRNA duplex-targeting GNA12, 40 nM of one TriFECTa siRNA duplex-targeting GNA13 and 10 nM of one TriFECTa siRNA duplex-targeting GNAQ were transfected into SaOS2 cells using the ViaFect transfection reagent (Promega Corporation, Madison, WI) under RNase-free conditions following the manufacturer's protocol.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

SaOS2 cells were plated at 0.7×10^5 cells/well in 24-well plates. Forty-eight hours after transfection with different amounts of Wnt16, cells were harvested and total RNA was prepared by the ReliaPrep RNA Cell Miniprep System (Promega Corporation, Madison, WI) to evaluate the expression of Wnt16 and osteoprotegerin (OPG). 12 and 24 h after transfection with Wnt16 plasmid DNA and target DsiRNAs, cells were harvested and RNA was isolated as mentioned above. For cDNA synthesis, an average of 160 ng total RNA was reverse transcribed to cDNA in 21 μl reactions using the SuperScript III First-Strand Synthesis System (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. qPCR was performed using the qPCR Core kits for SYBR Green I, No ROX (Eurogentec, Seraing, Belgium). Primer sequences available upon request. Each sample was analysed in triplicate and *B2M*, *HMBS* and *SDHA* were included as reference genes. Stability of

reference genes was verified using geNorm (Biogazelle, Ghent, Belgium) and efficiency of all primer pairs was checked with the qbase + software (Biogazelle, Ghent, Belgium). Expression of target and reference genes was quantified using qbase + software.

Enzyme-Linked Immunosorbent Assay (ELISA)

SaOS2 cells were plated at 0.5×10^5 cells/well in 24-well plates. Twenty-four hours after transfection with Wnt16 plasmid DNA and target DsiRNAs, cells were washed with PBS and trypsinized with TrypLE Express. Cell suspensions underwent three centrifugation steps (5 min, $600 \times g$, RT) after which the cell pellet was washed with cold PBS every time. Hereafter, cell lysis was attained by performing two freeze–thaw cycles. Next, lysed cells underwent centrifugation (15 min, $1000 \times g$, 4 °C) and supernatant was kept for further analysis. ELISA kits from MyBioSource (San Diego, CA) were ordered to measure protein levels of human GNA12 (#MBS9317428), GNA13 (#MBS9340640) and GNAQ (#MBS7234399). All ELISAs were performed according to the manufacturer's instructions. Each sample was analysed in duplicate and the NC1 DsiRNA with Wnt16 was used as a negative control. At last, to measure and correct for the total protein concentration of all samples, the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) was used.

Luciferase Reporter Assays with Gα12, Gα13 and Gαq Knockdown

SaOS2 cells were plated at 0.1×10^5 cells/well in 96-well plates 24 h prior to transfection. DsiRNAs were co-transfected with pCMV6-Entry-Wnt16 (40 ng), pRL-TK (2 ng) and pGL3-OT (30 ng), pGL4.30 (30 ng) or pGL4.44 (30 ng). As negative controls for each luciferase reporter assay with DsiRNAs, a NC1 scrambled duplex was co-transfected with empty pcDNA3.1 vector or Wnt16. 24 and 48 h after transfection, cells were lysed and firefly and Renilla luciferase activity were measured on a Glomax Multi + Luminometer (Turner Designs, Sunnyvale, CA) using the dual luciferase reporter assay system (Promega Corporation, Madison, WI) according to manufacturer's instructions under RNase-free conditions. The ratio of the firefly and Renilla luciferase measurement was calculated. Data of 48-h post-transfection are shown and expressed as mean values ± SD.

Statistical Analysis

All experiments were performed independently at least three times. The results shown in the figures represent the mean of triplicate measurements. For the luciferase reporter assays presented in Figs. 1 and 2, independent sample *T*-tests were

Fig. 1 Effect of Wnt16 on canonical WNT signalling in SaOS2 cells. **a** Wnt16 activates canonical WNT-signalling (TCF/LEF) in a dose-dependent manner in SaOS2 cells. **b** Upon activation by 80 ng of Wnt16, OPG expression is increased in SaOS2 cells *$p < 0.05$ compared between consecutive conditions
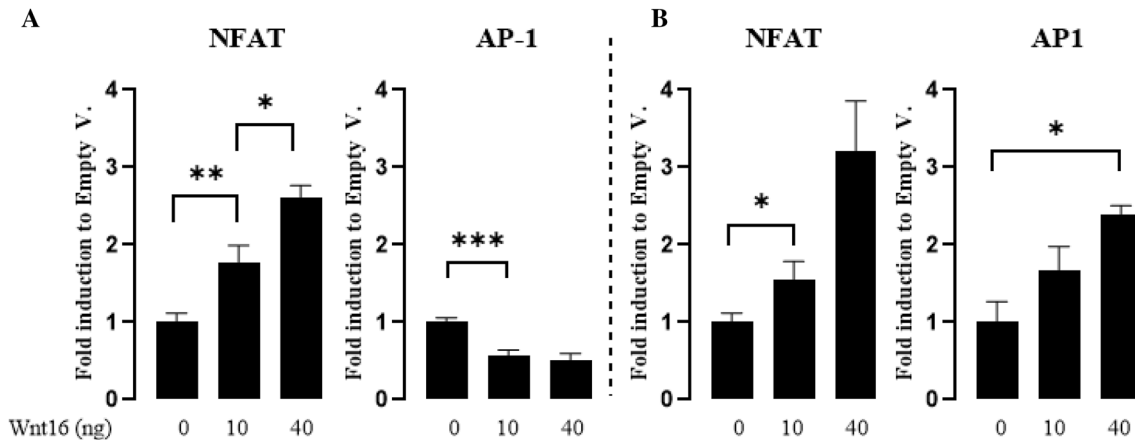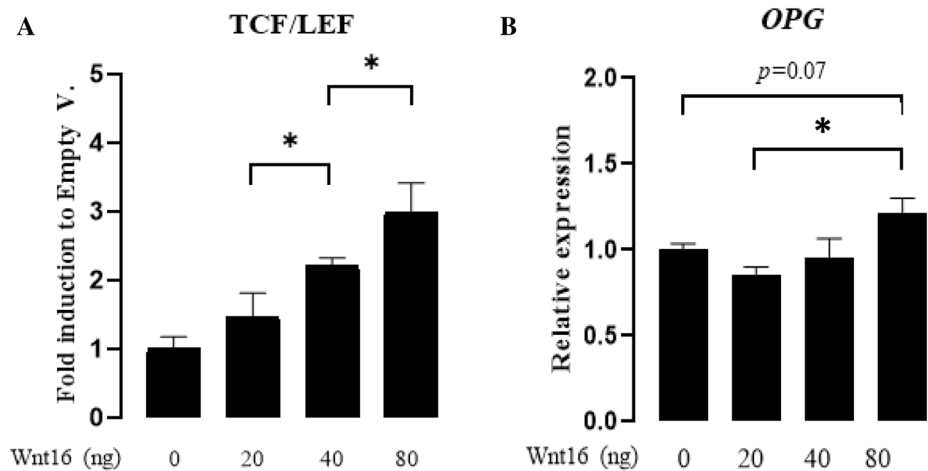
Fig. 2 Wnt16 modulates WNT/Ca^{2+} and WNT/PCP signalling differently in HEK293T and SaOS2 cells. **a** Wnt16 activates WNT/Ca^{2+} signalling (NFAT) in a dose-dependent manner in HEK293T cells, whereas WNT/PCP signalling (AP-1) was inhibited by Wnt16 in a dose-dependent manner. **b** A dose-dependent activation of both WNT/Ca^{2+} signalling and WNT/PCP signalling was observed in SaOS2 cells after Wnt16 treatment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

used to evaluate a dose-dependent effect between consecutive conditions. When no significant dose-dependent effect was observed, we investigated whether a significant increase was found between the control and the highest concentration. Statistical analysis of luciferase reporter assay data with siR-NAs (Fig. 3) was performed using one-way ANOVA, followed by a post hoc analysis with Dunnett's correction for multiple comparisons. The homoscedasticity assumption of the one-way ANOVA was visually inspected using boxplots and using Levene's test for equality of variances. Statistical tests were provided by the SPSS 25.0 software package (SPSS, Inc.).

Results

WNT16 Modulates WNT Signalling Differently in HEK293T and SaOS2 Cells

To investigate the effect of WNT16 on WNT signalling in osteoblasts, we performed WNT luciferase reporter assays in SaOS2 cells. In these osteoblast-like cells, we were able to confirm dose-dependent activation of WNT/β-catenin signalling by Wnt16 (Fig. 1). Furthermore, we
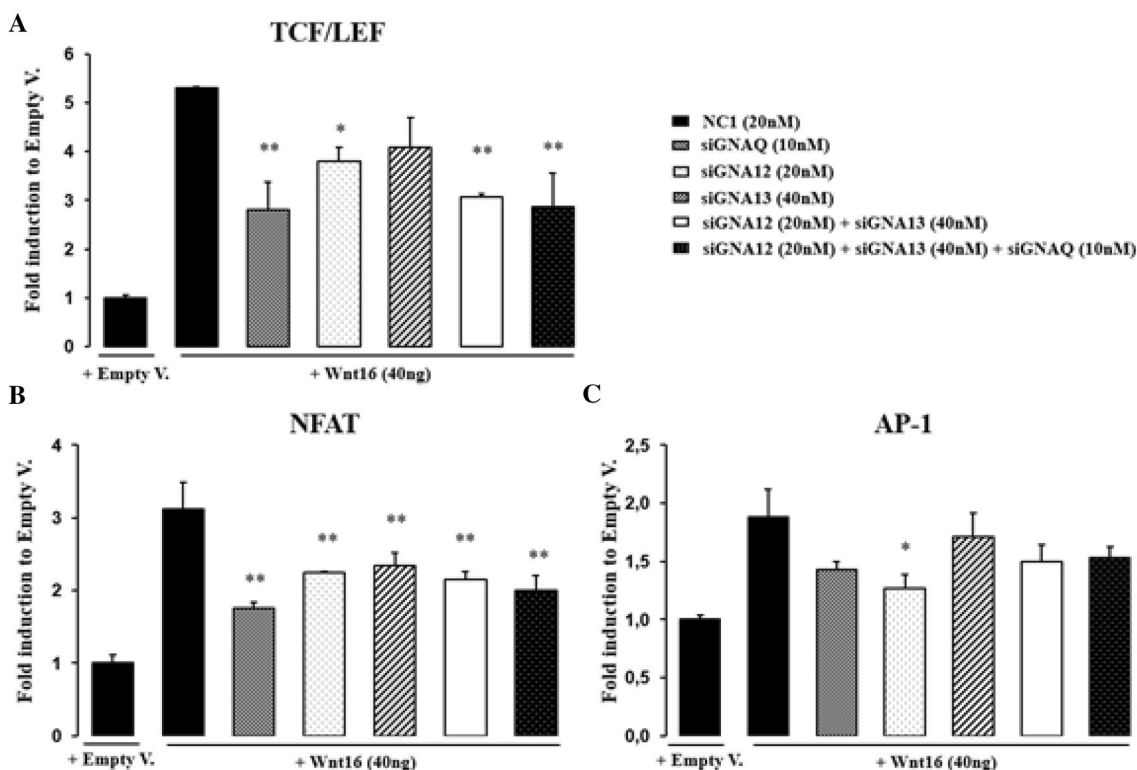
Fig. 3 Knockdown of Gαq (siGNAQ), Gα12 (siGNA12) and Gα13 (siGNA13) subunits alone or combined affects WNT signalling by Wnt16 in osteoblasts. **a** Activity of WNT/β-catenin signalling (TCF/LEF), **b** WNT/Ca²⁺ signalling (NFAT) and **c** WNT/PCP signalling (AP-1) by Wnt16, before and after knockdown of Gαq, Gα12 and/or

Gα13. Knockdown of all three Gα subunits alone and combined significantly decreased WNT/β-catenin signalling and WNT/Ca²⁺ signalling activity by Wnt16, whereas only knockdown of Gα12 alone significantly decreased WNT/PCP signalling. *$p < 0.05$, **$p < 0.01$ compared to WNT16 + NC1. *NC1* negative control DsiRNA

confirmed that the activation of the pathway by the highest dose of Wnt16 results in increased expression of OPG, a known target of the canonical Wnt signalling.

To explore and compare the effects of Wnt16 on noncanonical WNT signalling, a set of WNT luciferase reporter assays was performed in both HEK293T and SaOS2 cells (Fig. 2). To quantify non-canonical WNT signalling, different amounts of Wnt16 were co-transfected with a luciferase vector containing NFAT- or AP-1-responsive elements to monitor WNT/Ca²⁺ or WNT/PCP pathway activity. These experiments demonstrate that Wnt16 activates ($p < 0.05$) the NFAT-responsive element in a dose-dependent manner, but inhibits ($p < 0.01$) the AP-1 responsive element in a dose-dependent manner in HEK293T cells (Fig. 2a). In SaOS2 cells, both the NFAT and AP-1-responsive reporter assay, are significantly activated by Wnt16 (Fig. 2b). Altogether, Wnt16 activates WNT/Ca²⁺ signalling in HEK293T cells, but inhibits WNT/PCP signalling in a dose-dependent manner, whereas Wnt16 dose-dependently activates both noncanonical pathways in SaOS2 cells.

Selection and Knockdown of Gα12, Gα13 and Gαq in SaOS2 cells

Our data show that Wnt16 modulates the NFAT, AP-1 and TCF/LEF transcription factors in osteoblasts. Next, we aim to investigate the downstream mechanism of this activation. Based on the literature, Gα subunits from the Gαq and Gα12/13 subfamily have already been linked to modulation of these specific transcription factors, making these subunits interesting for further investigation [22, 23]. Members of the Gαi and Gαs subfamily are known to respectively decrease or increase cAMP levels. To possibly include or exclude these subunits in subsequent functional studies, the cAMP level was monitored after overexpression of Wnt16 in SaOS2 cells with a cAMP-sensitive luciferase reporter assay using the pCRE-Luc vector. Here, after transfecting different amounts of Wnt16, no increase or decrease of pCRE-Luc activity was detected, implying stable levels of cAMP (Supplemental Fig. 2). Since the NFAT, AP-1 and LEF/TCF transcription factors are activated by Wnt16, *GNA12* (NM_007353), *GNA13* (NM_006572) and *GNAQ*

(NM_002072) were targeted for knockdown, to verify the role of Gα12, Gα13 and Gαq, respectively, in the modulation of WNT signalling by Wnt16 in SaOS2 cells. Knockdown was verified on RNA and protein level by performing qRT-PCR and ELISA, respectively. Sufficient knockdown of Gα12, Gα13 and Gαq was achieved when transfecting 20 nM DsiGNA12, 40 nM DsiGNA13 and 10 nM DsiGNAQ. On mRNA level, knockdown was up to 70% 12 h and 24 h post-transfection for GNA12, GNA13 and GNAQ (Supplemental Fig. 3). ELISA confirmed levels of about 40% (GNA13, GNAQ) to 70% (GNA12) knockdown on protein level (Supplemental Fig. 4).

Gα Subunits are Intracellular Partners of Wnt16 in Osteoblasts

When performing the NFAT, AP-1 and LEF/TCF-responsive luciferase reporter assays in SaOS2 cells overexpressing Wnt16 with knockdown of Gαq alone, we detected a significant ($p < 0.01$) decrease in LEF/TCF and NFAT reporter activity compared to co-transfection of WNT16 with the NC1 negative control DsiRNA (Fig. 3). A decrease in AP-1 reporter activity was also detected, but did not reach significance levels. Overexpression of Wnt16 and knockdown of Gα12 alone also significantly decreased LEF/TCF ($p < 0.01$) and NFAT ($p < 0.05$) signalling activity and decreased AP-1 luciferase reporter activity ($p < 0.05$). Knockdown of Gα13 alone significantly ($p < 0.05$) decreased NFAT-signalling activity by Wnt16. Lastly, combined knockdown of Gα12-Gα13 or Gαq-Gα12-Gα13 similarly resulted in a significant decrease in LEF/TCF ($p < 0.01$) and NFAT ($p < 0.01$) signalling activity, but the decrease with regard to the positive control was not significantly greater than knockdown of only one Gα subunit.

Discussion

In the past years, *Wnt16* became an interesting target in the field of skeletal research, as it was identified as an essential regulator of the cortical bone compartment, with the ability to increase both cortical and trabecular bone mass and strength in vivo [12, 15–18, 20]. At the cellular level, osteoblasts are the principal source of WNT16, where they inhibit osteoclastogenesis in a direct (through osteoclasts) and indirect (through osteoblasts) manner [16]. There are indications that these beneficial effects are coming from the activation of both canonical and non-canonical WNT signalling in osteoblasts [16], but a clear model of WNT signalling by WNT16 is not yet depicted. Further elucidation of WNT16′s mechanism of action in osteoblasts could not only contribute to an improved understanding of WNT signalling in osteoblasts, but also identify putative mechanisms for the

therapeutic intervention of disorders with too little bone, like osteoporosis.

Therefore, we first compared the effect of Wnt16 on the different WNT-signalling pathways in two cell types, HEK293T and SaOS2 cells. Here, HEK293T cells were taken as a standard model, whereas SaOS2 cells are osteosarcoma cells that are considered a valid osteoblast-like cell model [24–26]. Previous findings indicated that WNT16 activates WNT/β-catenin signalling in MC3T3-E1 pre-osteoblastic cells [16, 21], whereas we formerly identified no activation of this pathway in HEK293T cells [10]. In this current study, the previously reported activation of WNT/β-catenin signalling by WNT16 in MC3T3-E1 cells is confirmed in SaOS2 cells, in a dose-dependent manner. Furthermore, we confirmed that high doses of Wnt16 can also increase the expression of OPG in SaOS2 cells, although less pronounced compared to what's reported for MC3T3-E1 cells previously [16]. Since both studies used a different method to deliver Wnt16 to the cells, it is hard to compare the effect of Wnt16 on the different cell types. However, the increase in OPG expression upon Wnt16 overexpression demonstrates that in SaOS2 cells, Wnt16 can act similarly as previously reported for MC3T3-E1 and that SaOS2 cells are a suitable model to study the effect of Wnt16 on Wnt signalling.

Next to canonical WNT signalling, we also showed differences in the effect of Wnt16 on non-canonical WNT/Ca^{2+} (NFAT) and WNT/PCP (AP-1) signalling in both cell models. WNT/Ca^{2+} signalling was activated by Wnt16 in both cell types, but WNT/PCP signalling was oppositely modulated. This suggests a specific relevance of WNT/PCP signalling activation by Wnt16 in osteoblasts. In SaOS2 cells, Wnt16 was able to dose-dependently activate both non-canonical WNT pathways. Previously, Movérare-Skrtic et al. also reported non-canonical activity of WNT16 in MC3T3-E1 cells, which was solely based on increased phosphorylated (thus activated) JNK and c-JUN [16]. Since JNK is known to activate the AP-1 transcription factor and AP-1 is composed of proteins belonging to c-JUN, c-FOS, ATF and JDP families, we were able to confirm this finding in SaOS2 cells. Altogether, studying the modulation of WNT signalling by Wnt16 in two different cell types clearly demonstrates that Wnt16 is a textbook example of a WNT that is able to differentially modulate WNT signalling in different cell types. While only activating the WNT/Ca^{2+} pathway and inhibiting WNT/PCP signalling in HEK293T cells, WNT16 activates WNT/β-catenin, WNT/Ca^{2+} and WNT/PCP signalling in osteoblasts. This suggests that the availability of certain receptors or intra/extracellular modulators varies markedly between cell types, which stresses the importance of investigating WNTs in suitable cell models to draw relevant conclusions.

As the starting point of WNT signalling, the spatiotemporal abundance of specific Fzd receptors is an important

determinant in this cell-receptor context and thus in WNT-signalling modulation by WNTs. Fzd receptors are GPCRs that can recruit G proteins composed of α, β and γ subunits, with specific intracellular effects [27]. Based on the literature and our signalling studies, we designated Gαq and Gα12/13 as most interesting for knockdown experiments. These studies point out an essential upstream role of all three Gα subunits in the WNT/β-catenin and WNT/Ca^{2+}-signalling activity by Wnt16 in osteoblasts, and of Gα12 in WNT/PCP signalling, illustrating a novel mechanism of interplay between the different WNT-signalling pathways in osteoblasts. The correlation between active Gαq, an increase in Ca^{2+} levels and subsequent NFAT activation has been well-documented in other cell types or research fields (Fig. 4) [22, 23]. Similarly, Gα12 is well-known to activate RhoA through a direct interaction with Rho guanine nucleotide exchange factors (RhoGEFs), subsequent activation of Rho-dependent kinase (ROCK), JNK and eventually AP-1 [22, 23, 28]. To clarify the findings on WNT/β-catenin signalling, there are several hypotheses (Fig. 4). We found in the literature that active Gαq can inactivate GSK-3β, resulting in the cytoplasmic stabilization and accumulation of β-catenin and WNT/β-catenin signalling activity. Next, Gαq, Gα12 and Gα13 were all three reported to interact with

the cytoplasmic domain of cadherins, where a great part of the cellular β-catenin pool is anchored. Binding of these Gα subunits was hence proven to be a nonstandard mechanism for β-catenin release, stabilization and WNT/β-catenin signalling activation [29–31]. N- and E-cadherin are reported as the most relevant cadherins in osteoblasts and we only found expression of N-cadherin, not E-cadherin, in SaOS2 cells (data not shown). Also in this regard, it was previously demonstrated that WNT16, through WNT/PCP-signalling activity in leukemia cells, was associated with an increased N-cadherin expression on the membrane [32]. This correlation between WNT/PCP signalling and N-cadherin expression has been reported in other studies as well [33].

In conclusion, we have shown that Wnt16 modulates WNT signalling differently in disparate cell types. In osteoblasts, Wnt16 activates WNT/β-catenin, WNT/Ca^{2+} and WNT/PCP signalling. Upon extracellular binding of Wnt16 to its Fzd receptor, we demonstrate that specific Gα subunits play an orchestrating role in the downstream activity of Wnt16 in osteoblasts, resulting in a model of Wnt16-Gα signalling in osteoblasts (Fig. 4). In the future, additional studies are needed to demonstrate whether this mechanism is specific for WNT16 signalling or relevant for all other WNT ligands as well. In addition, further investigation of
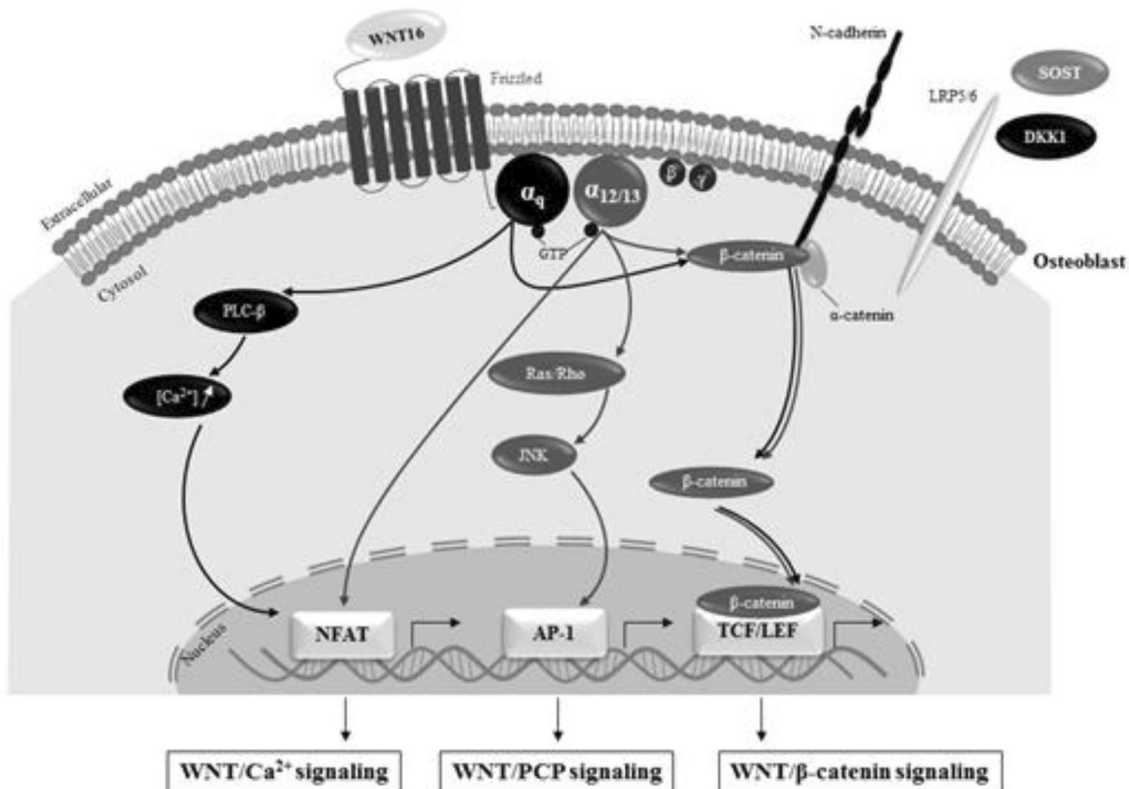
Fig. 4 Wnt16-Gα signalling in osteoblasts: a model. Wnt16 activates WNT/β-catenin signalling (TCF/LEF), WNT/Ca^{2+} signalling (NFAT) and WNT/PCP signalling (AP-1) in osteoblasts with the intracellular aid of the Gαq, Gα12 and Gα13 subunits

the specific consequences of Wnt16-Gα signalling for osteoblast function and its communication with the osteoclast can further contribute to a better understanding of the in vivo data and help to explore therapeutic options of WNT16 for bone disorders like osteoporosis. In this regard, this study also highlights the Fzd receptor as an essential link towards the beneficial effects of WNT16. Generally, GPCRs have already been pointed out as desirable therapeutic targets in the field of oncology, type 2 diabetes, neurodegenerative and cerebrovascular diseases and multiple sclerosis due to their diversity, cell-specific expression patterns and 'druggability' [27, 34–37]. As for osteoporosis, more research is required to further verify the potential of the Fzd receptors as putative therapeutic target.

Authors contributions Study design: GH, EB and WVH. Study conduct: GH. Data analysis: GH, EB, MV and EF. Data interpretation: all authors. Drafting manuscript: GH and MV. Revising manuscript: all authors. Approving manuscript for publication: all authors. GH takes responsibility for the integrity of the data analysis.

Compliance with Ethical Standards

Conflict of interest Gretl Hendrickx, Eveline Boudin, Marinus Verbeek, Erik Fransen, Geert Mortier and Wim Van Hul declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

References

1. Boudin E et al (2013) The role of extracellular modulators of canonical Wnt signaling in bone metabolism and diseases. Semin Arthritis Rheum 43(2):220–240
2. Dann CE et al (2001) Insights into Wnt binding and signalling from the structures of two Frizzled cysteine-rich domains. Nature 412(6842):86–90
3. Dijksterhuis JP, Petersen J, Schulte G (2014) WNT/Frizzled signalling: receptor-ligand selectivity with focus on FZD-G protein signalling and its physiological relevance: IUPHAR Review 3. Br J Pharmacol 171(5):1195–1209
4. Boudin E et al (2016) Genetic control of bone mass. Mol Cell Endocrinol 432:3–13
5. Estrada K et al (2012) Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet 44(5):491–501
6. Kemp JP et al (2016) Genome-wide association study of bone mineral density in the UK Biobank Study identifies over 376 loci associated with osteoporosis. In: American Society of Bone and Mineral Research, annual meeting 2016 abstracts
7. Hendrickx G, Boudin E, Van Hul W (2015) A look behind the scenes: the risk and pathogenesis of primary osteoporosis. Nat Rev Rheumatol 11(8):462–474
8. Chesi A et al (2015) A trans-ethnic genome-wide association study identifies gender-specific loci influencing pediatric aBMD and BMC at the distal radius. Hum Mol Genet 24(17):5053–5059
9. Garcia-Ibarbia C et al (2013) Missense polymorphisms of the WNT16 gene are associated with bone mass, hip geometry and fractures. Osteoporos Int 24(9):2449–2454
10. Hendrickx G et al (2014) Variation in the Kozak sequence of WNT16 results in an increased translation and is associated with osteoporosis related parameters. Bone 59:57–65
11. Koller DL et al (2013) Meta-analysis of genome-wide studies identifies WNT16 and ESR1 SNPs associated with bone mineral density in premenopausal women. J Bone Miner Res 28(3):547–558
12. Medina-Gomez C et al (2012) Meta-analysis of genome-wide scans for total body BMD in children and adults reveals allelic heterogeneity and age-specific effects at the WNT16 locus. PLoS Genet 8(7):e1002718
13. Moayyeri A et al (2014) Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Hum Mol Genet 23(11):3054–3068
14. Zhang L et al (2014) Multistage genome-wide association meta-analyses identified two new loci for bone mineral density. Hum Mol Genet 23(7):1923–1933
15. Zheng HF et al (2012) WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. PLoS Genet 8(7):e1002745
16. Moverare-Skrtic S et al (2014) Osteoblast-derived WNT16 represses osteoclastogenesis and prevents cortical bone fragility fractures. Nat Med 20(11):1279–1288
17. Alam I et al (2017) Bone mass and strength are significantly improved in mice overexpressing human WNT16 in osteocytes. Calcif Tissue Int 100(4):361–373
18. Alam I et al (2016) Osteoblast-specific overexpression of human WNT16 increases both cortical and trabecular bone mass and structure in mice. Endocrinology 157(2):722–736
19. Moverare-Skrtic S et al (2015) The bone-sparing effects of estrogen and WNT16 are independent of each other. Proc Natl Acad Sci USA 112(48):14972–14977
20. Gori F et al (2015) A new WNT on the bone: WNT16, cortical bone thickness, porosity and fractures. Bonekey Rep 4:669
21. Jiang Z et al (2014) Wnt16 is involved in intramembranous ossification and suppresses osteoblast differentiation through the Wnt/beta-catenin pathway. J Cell Physiol 229(3):384–392
22. Dorsam RT, Gutkind JS (2007) G-protein-coupled receptors and cancer. Nat Rev Cancer 7(2):79–94
23. Worzfeld T, Wettschureck N, Offermanns S (2008) G(12)/G(13)-mediated signalling in mammalian physiology and disease. Trends Pharmacol Sci 29(11):582–589
24. McQuillan DJ, Richardson MD, Bateman JF (1995) Matrix deposition by a calcifying human osteogenic sarcoma cell line (SAOS-2). Bone 16(4):415–426
25. Murray E et al (1987) Characterization of a human osteoblastic osteosarcoma cell line (SAOS-2) with high bone alkaline phosphatase activity. J Bone Miner Res 2(3):231–238
26. Rodan SB et al (1987) Characterization of a human osteosarcoma cell line (Saos-2) with osteoblastic properties. Cancer Res 47(18):4961–4966
27. Schulte G, Bryja V (2007) The Frizzled family of unconventional G-protein-coupled receptors. Trends Pharmacol Sci 28(10):518–525

28. Riobo NA, Manning DR (2005) Receptors coupled to hetero-trimeric G proteins of the G12 family. Trends Pharmacol Sci 26(3):146–154

29. Fujino H, Regan JW (2001) FP prostanoid receptor activation of a T-cell factor/beta-catenin signaling pathway. J Biol Chem 276(16):12489–12492

30. Meigs TE et al (2002) Galpha12 and Galpha13 negatively regulate the adhesive functions of cadherin. J Biol Chem 277(27):24594–24600

31. Meigs TE et al (2001) Interaction of Galpha 12 and Galpha 13 with the cytoplasmic domain of cadherin provides a mechanism for beta-catenin release. Proc Natl Acad Sci USA 98(2):519–524

32. Nygren MK et al (2009) beta-catenin is involved in N-cadherin-dependent adhesion, but not in canonical Wnt signaling in E2A-PBX1-positive B acute lymphoblastic leukemia cells. Exp Hematol 37(2):225–233

33. Yang L et al (2007) Rho GTPase Cdc42 coordinates hematopoietic stem cell quiescence and niche interaction in the bone marrow. Proc Natl Acad Sci USA 104(12):5091–5096

34. Du C, Xie X (2012) G protein-coupled receptors as therapeutic targets for multiple sclerosis. Cell Res 22(7):1108–1128

35. Guerram M, Zhang LY, Jiang ZZ (2016) G-protein coupled receptors as therapeutic targets for neurodegenerative and cerebrovascular diseases. Neurochem Int 101:1–14

36. Lappano R, Maggiolini M (2011) G protein-coupled receptors: novel targets for drug discovery in cancer. Nat Rev Drug Discov 10(1):47–60

37. Reimann F, Gribble FM (2016) G protein-coupled receptors as new therapeutic targets for type 2 diabetes. Diabetologia 59(2):229–233