Nanoparticle and nanotopography-induced activation of the Wnt pathway in bone regeneration

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Abstract:

Background and Aims: Recent research has focused on developing nanoparticle and nanotopography-based technologies for bone regeneration. The Wnt signalling pathway has been shown to play a vital role in this process, in particular in osteogenic differentiation and proliferation. The exact mechanisms by which nanoparticles and nanotopographies activate the Wnt signalling pathway however are not fully understood. This review aimed to elucidate the mechanisms by which nano-scale technologies activate the Wnt signalling pathway during bone regeneration.

Methods: The terms "Wnt", "bone", and "nano*" were searched on PubMed and Ovid with no date limit. Only original research articles related to Wnt signalling and bone regeneration in the context of nanotopographies, nanoparticles or scaffolds with nanotopographies/nanoparticles were reviewed.

Results: The primary mechanism by which nanoparticles activated the Wnt pathway was by internalisation via the endocytic pathway or diffusion through the cell membrane, leading to accumulation of non-phosphorylated β-catenin in the cytoplasm and subsequently downstream osteogenic signalling (e.g. upregulation of RUNX2). The specific size of the nanoparticles and the process of endocytosis itself has been shown to modulate the Wnt-β-catenin pathway. Nanotopographies were shown to directly activate frizzled receptors, initiating Wnt/β-catenin signalling. Additional studies showed nanotopographies to activate the Wnt/Ca^{2+} dependent and Wnt/planar cell polarity pathways via nuclear factor of activated T-cells, and α5β1 integrin stimulation. Finally, scaffolds containing nanotopographies/nanoparticles were found to induce Wnt signalling via a combination of ion release (e.g. lithium, boron, lanthanum and icariin) which inhibited GSK-3β activity, and via similar mechanisms to the nanotopographies.

Conclusion: This review concludes that nanoparticles and nanotopographies cause Wnt activation via several different mechanisms, specific to the size, shape and structure of the nanoparticles or nanotopographies. Endocytosis-related mechanisms,

integrin signalling and ion release were the major mechanisms identified across nanoparticles, nanotopographies and scaffolds respectively. Knowledge of these mechanisms will help develop more effective targeted nanoscale technologies for bone regeneration.

Impact statement: Nanoparticles and nanotopographies can activate the Wnt signalling pathway, which is essential for bone regeneration. This review has identified that activation is due to endocytosis, integrin signalling and ion release, depending on the size, shape, and structure of the nanoparticles or nanotopographies. By identifying and further understanding these mechanisms, more effective nanoscale technologies that target the Wnt signalling pathway can be developed. These technologies can be used for the treatment of non-union bone fractures, a major clinical challenge, with the potential to improve the quality of life of millions of patients around the world.

1. Introduction

The global prevalence of bone fractures is estimated to be around 9 million annually, with the risk of developing non-union healing thought to be between 1.9-4.9% [1]. Non-union healing is a painful condition with an extended healing period of around 20-30 weeks, with some non-healing fractures taking more than a year to fully heal [2]. This extended healing time results in morbidity and affects patient quality of life. Many treatment concepts have been developed to enhance bone regeneration in non-union healing, such as the use of autologous bone grafts, mesenchymal stem cells and growth factors. One promising approach exploits mechanisms of mechanotransduction (conversion of mechanical stimuli into an intracellular signal) [1]. Mechanical stimulus has been shown to play a significant role in directing mesenchymal cell fate towards an osteogenic lineage through the Wingless-related integration site (Wnt) signalling pathway [2–4].

The Wnt signalling pathway controls many aspects of cell biology, such as cell polarity, fate, migration and proliferation. 19 Wnt genes encode the Wnt ligands associated with cell membranes and extracellular matrix (ECM). These ligands bind to the receptors on the cell surface and activate the Wnt pathway by triggering intracellular signalling cascades. Before binding to the receptors, these proteins undergo palmitoylation in the endoplasmic reticulum in the presence of acyltransferase porcupine [5]. These modified Wnt proteins are released from the cells through secretory vesicles, and this process is controlled by Wntless/Evi (evenness interrupted) proteins. The released Wnt proteins then bind to the frizzled receptor (Fzd) with the low-density lipoprotein receptor-related proteins 5 and 6 (LRP5, LRP6) or receptor-tyrosine-kinase-like orphan receptor (ROR) acting as co-receptors. The co-receptors assist the receptor-ligand binding and further activate the intracellular signalling cascade.

There are three intracellular pathways in Wnt signalling: (1) The canonical Wnt pathway, otherwise known as the β-catenin dependent pathway; (2) the planar cell polarity (PCP) pathway; and (3) the Wnt/Ca^{2+} pathway (Fig 1).

Canonical Wnt signalling results in the accumulation and translocation of β-catenin into the nucleus and activation of transcription factors, which upregulate specific genes [5]. In the case of osteogenic-related genes, the upregulation of alkaline phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX2*), osterix (*SP7*), osteopontin (*OPN*) and osteocalcin (*OCN*) are commonly reported. During activation of the canonical pathway by the Wnt proteins, the proteins bind to the receptor and activate the dishevelled protein (Dvl), which leads to the disintegration of the Glycogen synthase kinase 3 beta (GSK-3β) complex (Axin, GSK-3β, casein kinase1 and adenomatous polyposis coli). This prevents phosphorylation of β-catenin (an adherens junction-associated protein that enables cell-cell adhesion), ultimately preventing its ubiquitination and degradation. As a result, there is increased accumulation of cytoplasmic β-catenin. This accumulated β-catenin subsequently translocates into the nucleus and binds with T cell factor/lymphoid enhancer factor (TCF/LEF) and results in downstream activation of transcription factors [5]. In the absence of Wnt ligands, the β-catenin in the cytoplasm is phosphorylated by the GSK-3β complex, which results in beta-transducin repeat containing E3 Ubiquitin Protein Ligase (BTrCP) ubiquitinating the phosphorylated β-catenin. This targets the β-catenin for proteosomal degradation.

The inhibitors of Wnt signalling are sclerostin (SOST) (a potent inhibitor of bone formation) and Dickkopf (DKK1) (Fig 1). These inhibitors bind to LRP5/LRP6 and thus antagonise canonical Wnt signalling. An upregulation of sclerostin and Dickkopf has been reported during bone resorption [6]. It is thought that osteocytes also regulate osteoblast activity by secreting sclerostin and Dickkopf. Mechanical force stimulates osteocytes to downregulate sclerostin production, whilst in unloaded bone, sclerostin production has been shown to increase [7].

The canonical pathway is thought to modulate the entire osteoblastic lineage, inhibiting mesenchymal cell commitment to other lineages. WNT/β-catenin signalling also indirectly inhibits osteoclast differentiation and bone resorption through increased secretion of osteoprotegerin (OPG) by osteocytes. Osteoprotegerin is the decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL), preventing the interaction of RANKL with receptor activator of nuclear factor kappa-B (RANK) on pre-

osteoclasts, a process necessary for mature osteoclast differentiation [7]. The Wnt ligands however can directly affect osteoclasts in several ways. In the early stages of bone resorption, Wnt3a (WNT/β-catenin signalling agonist) favours preosteoclast proliferation, but in the later stages, it inhibits osteoclastogenesis. Wnt5a expressed by osteoblasts stimulates differentiation of osteoclast precursors; however, at the end of the resorption phase, osteoclasts in turn stimulate the local differentiation of osteoblasts by also secreting Wnt ligands [8].

Although the canonical pathway has been more widely studied for bone regeneration, it should be noted that the non-canonical Wnt signalling pathways also play vital roles in the osteogenic differentiation of mesenchymal stem cells. The non-canonical Wnt pathway is divided into the PCP pathway and the Wnt/Ca^{2+} pathway.

The activation of the PCP pathway causes cytoskeletal rearrangement and leads to asymmetrical organisation, affecting cell polarisation and fate. Similar to the canonical pathway, the receptor for the PCP pathway is the Fzd receptor; however, in this case, the co-receptor is the receptor tyrosine kinase-like orphan receptor (ROR). The activation of Fzd causes binding and activation of Dvl. The activated Dvl then forms a complex with the Dishevelled associated activator of morphogenesis 1 (DAAM1). This complex further activates small G protein Rho, which in turn activates Rho-associated kinase (ROCK), a molecule responsible for cell movement and polarity. Alternatively, the activated Dvl forms a complex with Ras-related C3 botulinum toxin substrate (RAC) and activates jun N-terminal kinase (JNK), upregulating osteogenic transcription factors like RUNX2 (Fig 1)[8].

The Wnt5a protein can also activate the Wnt/Ca^{2+} non-canonical pathway. Similar to the PCP pathway, Wnt5a binds to Fzd and ROR however in the Wnt/Ca^{2+} non-canonical pathway, phospholipase C (PLC) and dystroglycan 1 (DAG) are activated instead of RAC and DAAM1. This further activates many cascades via G-protein linked protein kinase C (PKC) and inositol 1,4,5-trisphosphate, type 3 (IP3) generation. The activation of IP3 causes an increase in intracellular Ca^{2+} and downstream, activates NFAT. This

increase in Ca^{2+} inhibits peroxisome proliferator-activated receptor-γ (PPAR-γ) and directs the cell towards osteogenic differentiation [5].

Both canonical and non-canonical pathways play critical roles in osteogenic differentiation and endocrine factors that also regulate bone turnover, such as parathyroid hormones (PTH), have been shown to crosstalk with the Wnt signalling pathway [9]. There are multiple mechanisms by which PTH and Wnt/β-catenin pathways crosstalk (Fig 1). One hypothesis is that PTH decreases the expression of sclerostin and Dickkopf. The second hypothesis is that PTH binds to the PTH related peptide 1 receptor (PTH1R), a G-protein coupled receptor, and stabilises β-catenin. In the absence of Wnt ligands, it is thought that PTH binds to LRP5 and triggers the pathway. PTH1R, in the presence of Wnt signals, directs progenitor cells towards osteoblast differentiation [8], driving bone regeneration.

The use of nanotechnology (materials that possess at least one dimension between 1-100 nm) to encourage bone healing, has gained significant interest over the past decade. A review by Griffin et al. (2017) highlighted the potential interactions between nanomaterials and cells and their significance in bone tissue engineering. Nanomaterials demonstrate nanoscale roughness, changes in wettability and increased surface area. These nanotopographical cues control osteogenic differentiation through cell adhesion and cytoskeletal organisation, thereby promoting bone regeneration. Geometric parameters at a nanoscale, such as size, shape and interconnectivity of nanostructures also play an important role in this process, particularly when nanostructures mimic the extracellular matrix (ECM) of bone [10].

Gong et al. (2015) discussed how nanostructures provide structural support for bone architecture and regulate cell differentiation. The review also illustrated that nanotopographies can alter the clustering of integrins, increase the formation of focal adhesions and thus cause cytoskeletal reorganisation. These changes regulate mechanical signals and direct the cells toward osteogenic differentiation [11]. The author illustrated that the enhanced osteogenic differentiation was mainly through the Rho-associated protein kinase (ROCK) pathway, which is one of the Wnt/PCP pathways.

The cell shape is directed by the nanotopography geometry, with the width:spacing ratio playing a vital role in cell differentiation. The cells are directed towards osteogenic differentiation when the width:spacing ratio is between 1:1 and 1:3. Thus, nanotechnology can be used to design nanostructures that mimic the hierarchical design of bone at the nano and microscale.

Additionally, nanoparticles have also demonstrated significant promise for bone regeneration. The majority of studies in this area have exploited the benefits of utilising nanoparticles for drug delivery, such as high drug loading efficiency, long circulation half-life and the ability of nanoparticles to diffuse into tissues. In particular for bone regeneration, nanoparticles have successfully been used for the delivery of drugs (e.g. bisphosphonates), growth factors (e.g. BMP-2) and gene delivery (e.g. DNA, siRNAs, microRNAs), using either biodegradable (e.g. PLA, PLGA) or non-degradable materials (e.g. hydroxyapatite, gold, silica) [12]. The success of this approach is highlighted by the growing number of clinical trials in this area.

The ability to encourage bone regeneration, either through modulating cell behaviour or through the delivery of therapeutics, demonstrates the significant potential for nanotechnology to treat bone diseases, such as osteoporosis and non-union fractures, where existing treatments are inadequate. The development of such technologies will not only improve treatment outcomes for millions of patients globally, alleviating significant burdens on both patients and health services, but will also result in significant economic benefits.

In the past decade, substantial research has focussed on developing Wnt activating nanotechnologies for bone regeneration; however, the mechanism of activation of the pathway is less understood [11]. This review therefore aimed to elucidate the mechanism by which nanotechnology, in particular nanoparticles, nanotopographies and scaffolds, activate the Wnt signalling pathway during bone regeneration.

2. Methods

The PRISMA checklist was used as a guide for this review [13]. The research of interest related to the activation of the Wnt signalling pathway by nanotopographies or nanoparticles for bone regeneration.

2.1. Search strategy

Ovid MEDLINE®, Embase and PubMed were searched with no time restrictions up until the 1st December 2022, using the terms "Wnt" AND "bone" AND "Nano*". Duplicates were removed, and the remaining full-text articles were assessed and subjected to inclusion and exclusion criterion.

The inclusion criteria were as follows:

1. Original research articles.
2. Articles related to Wnt signalling and bone regeneration.
3. Articles related to nanotopographies (such as those achieved through mechanical, laser or chemical modification of metal surfaces, as well as coatings consisting of nanoscale structures, such as nanostructured hydroxyapatite, nanorods, nanotubes, etc…), nanoparticles (such as gold, hydroxyapatite, iron, silica, etc…) or scaffolds (such as porous metal, hydroxyapatite-based, bioactive glass, polymer scaffolds, etc…) with nanotopographies or nanoparticles.
4. *In vitro*, *in vivo* and *ex vivo* studies.

Exclusion criteria:

1. Review articles.
2. Non-English articles.
3. Articles related to cancer, oncogenes, metastasis and tumours as these conditions are not directly related to healthy bone regeneration.
4. Delivery of siRNA, mRNA, miRNA, DNA and other genes have been excluded as such studies aim to modify the behaviour of cells through genetic means, which may mask the mechanisms by which nanoparticles, nanotopographies and scaffolds directly modulate the Wnt pathway.
5. Conference abstracts.

6. Articles not related to bone.

A total of 551 articles were identified from the searches, and after removing the duplicates, a total of 499 articles were screened for inclusion and exclusion criteria. Titles and abstracts were initially analysed for relevance and if unclear, the full text articles were reviewed. 467 articles were excluded based on the exclusion criteria. The majority of these articles were excluded due to their focus on gene delivery or bone cancer or due to no experimental data on Wnt pathway activation being presented. Full-text assessments were performed for the remaining 32 articles, and a further 2 articles were excluded due to their relation to gene delivery and gene splicing. Finally, 30 articles were included in the review (Fig 2).

3. Results and discussion

The reviewed literature was categorised based on the nature of the nanotechnology and are discussed below under the subheadings of nanoparticles, nanotopographies and scaffolds.

3.1. Nanoparticles

Gold nanoparticles are highly biocompatible, have unique optical properties and have surfaces that can be functionalised. Liang et al. (2019) demonstrated that gold nanoparticles loaded with hydroxyapatite (HA-AU) are capable of increasing human bone marrow-derived mesenchymal stem cell (hBMSC) osteogenic differentiation and mineralisation under osteogenic conditions and attributed this observation to activation of the Wnt pathway. The HA-AU nanoparticles were rod shaped, measuring approximately 80–100 nm in length and 20–30 nm in width, with concentrations of 100 μg/mL or less, not significantly impacting hBMSC viability ($p>0.05$) [14].

The proposed mechanism of accelerated osteogenesis was through Wnt activation, caused by the internalisation of the nanoparticles. The HA-AU nanoparticles were

presumed to enter the cells via the endocytic pathway as they were detected inside endosomal vesicles inside the cell (Fig 3A) [14]. This is likely through a clathrin-mediated mechanism, as elliptical nanoparticles, similar to those developed by Liang et al. (2019), exhibit higher clathrin-coating, internalisation and transportation efficacy [15]. Western blots and qPCR showed an increase in β-catenin in HA-AU treated hBMSCs. Treating hBMSCs with HA-AU combined with 10 μM ICG-001 (a Wnt/β-catenin pathway inhibitor) however, decreased ALP production and calcium deposition indicating the Wnt/β-catenin pathway to play a role in the nanoparticle induced osteogenic differentiation process.

Blitzer et al, (2006) has shown that blocking endocytosis reduces β-catenin stabilisation during both mammalian (Wnt-3A) and *Drosophila* (Wg) Wnt signalling [16]. Conversely, activation of Wnt signalling using lithium (a well-known agonist of the canonical Wnt pathway, which inhibits GSK3β activity, allowing stabilisation of β-catenin in the cytosol) [17] has been shown to induce rapid endocytosis [18]. This highlights a strong link between endocytosis and Wnt signalling that has yet to be fully elucidated. Endocytosis has been proposed to regulate Wnt/β-Catenin signalling via four potential mechanisms: sequestration of GSK-3β to limit β-catenin degradation; clearance of ubiquitin ligases; early endosomal acidification of Lrp6 to activate the pathway; and by stabilization of Dvl to facilitate signalling [19]. Studies have also shown that binding of multiple Wnt proteins to Fzd receptors leads to clustering and endocytosis of a multimeric complex consisting of several receptors and ligands, known as the signalosome which is 40-100 nm in diameter [19–23]. Given the size of the nanoparticles (approximately 80–100 nm in length and 20–30 nm in width) used by Liang et al. (2019), it is possible that the process of clathrin-dependent endocytosis of the nanoparticles, similar in size to the Wnt signalosome, may cause the activation of the Wnt/β-catenin pathway. Interestingly, a study by Rejman et al. (2004) demonstrated that an upper size limit of approximately 200 nm exists for clathrin-mediated endocytosis, with larger particles being internalised via caveolae-mediated mechanisms [24]. These differences in endocytic mechanisms based on particle size may influence the levels of Wnt activation, however this hypothesis is yet to be tested.

A similar study by Zhang et al. (2021) found 45nm PEGylated gold nanoparticles (AuNPs) significantly increased alkaline phosphatase activity and mineralisation in MC3T3-E1 cells as well as hBMSCs and rat bone marrow mesenchymal stem cells (rBMSCs) [25]. Western blot analysis indicated that the Wnt/β-catenin pathway was the cause behind this observation due to elevated β-catenin and phosphorylated GSK-3β levels. Interestingly the effect was dampened as the nanoparticle diameter decreased (e.g. 4 and 18 nm), further highlighting the importance of nanoparticle size in activating Wnt signalling.

Choi et al. (2015) also proposed the internalisation of chitosan-conjugated AuNPs into the cell as the mechanism of activation of the Wnt pathway. Treating human adipose-derived mesenchymal stem cells (hADMSCs) with 0.5ppm and 1ppm chitosan-conjugated AuNPs did not influence cell viability, however under osteogenic conditions, significantly increased mineralisation and osteogenic marker expression (alkaline phosphatase, bone sialoprotein and osteocalcin) and attributed this to the Wnt/β-catenin pathway [26]. The study proposed that the AuNPs may directly diffuse through the cell membrane due to the smaller size of the nanoparticles (~17 nm, Fig 3B). Although embedding of the AuNPs in the cell wall was observed with transmission electron microscopy images, it is unlikely that a particle greater than 4 nm is capable of passively diffusing across the cell membrane.

Zhang et al. (2019) hypothesised that mild localised heat (40−43 °C) induced using photothermal therapy (PTT) on nanoparticles (~50 nm) within cells could accelerate cell proliferation and bone regeneration through the Wnt pathway [27]. Porous gold-palladium alloy nanoparticles (pAuPds) were synthesised with an absorption band of 705 nm. After entering the cells, the pAuPds were activated using a near-infrared laser to produce mild localised heat. The *in vitro* results demonstrated that mild localised heat accelerated cell proliferation and bone regeneration in pre-osteoblastic MC3T3-E1 cells. An *in vivo* study was also performed using an 8 mm critical-sized cranial defect rat model. The optimum concentration of pAuPds used was 100 μg/mL and when

combined with a laser treatment, achieved approximately 97% defect closure in 6 weeks, compared to 20% for the PBS control. RNA sequencing analysis showed an increase in Wnt genes (*Wnt10b* and *A1p1*) in the PTT group. This finding is contrary to those by Zhou et al. (2020) who applied 41 °C heat directly to intestinal epithelial cells and observed a decrease in Wnt/β-catenin activation [28]. The differences in these findings can be explained by the fact that Zhang et al. (2019) applied localised heat within the cell cytosol, whilst Zhou et al. (2020) simply exposed the cells to higher incubation temperatures, which would result in an apoptotic response and down-regulation of Wnt signalling. There are also likely differences in the response to heat between the pre-osteoblast cell line (MC3T3-E1) used by Zhang et al. (2019) and the intestinal porcine enterocytes (IPEC-J2) used by Zhou et al. (2020). Additionally, treatment of cells with gold nanoparticles, as previously discussed, will likely activate the Wnt/β-catenin pathway without requiring thermal treatment.

Iron nanoparticles are proposed to activate Wnt signalling in a similar manner to gold nanoparticles. Jia et al. (2019) synthesised mesoporous silica-coated magnetic (Fe_3O_4) nanoparticles (M-MSNs, Fig 3C) and treated mesenchymal stem cells (MSCs) with 0, 10, 50, 100 and 300 μg/mL [29]. The authors demonstrated no change in viability, however in osteogenic induction medium (20 mM β-glycerophosphate, 50 μM L-ascorbic acid-2-phosphate, and 1 nM dexamethasone), an increase in alkaline phosphatase staining after 7 days and increased mineralisation after 14 days were observed. The authors demonstrated not only increased β-catenin mRNA and protein levels, but also high levels of phosphorylated GSK-3β. The results were confirmed in a rat distraction osteogenesis model, which demonstrated accelerated bone repair and a greater presence of β-catenin. The activation of the Wnt pathway was thought to be through internalisation of the M-MSNs (similar to the previously discussed gold nanoparticles) and further through internal release of silica ions, which act as an inhibitor of GSK-3β, thus preventing phosphorylation of β-catenin and its degradation (Fig 3C).

He et al. (2020) proposed that iron oxide nanoparticles could be added to graphene oxide (GO) to reduce the cytotoxicity of GO. This study showed that magnetic GO

(MGO) was biocompatible and accelerated the osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells (rBMSCs) compared to untreated GO [30]. The results demonstrated an increase in Wnt related genes and osteogenic genes amongst the MGO treated group: however, desirable outcomes were noted only in osteogenic induction medium, similar to those reported by Liang et al. (2019), Choi et al. (2015) and Jia et al. (2019). The study stated that the osteogenic differentiation was concentration-dependent, and that MGO concentrations beyond the optimum level of 0.1 μg/mL could be cytotoxic [31,32]. El-Kady et al. (2016) and Scarpa et al. (2018) also demonstrated that nanoparticles could be used to carry small molecules like lithium ions and 6-bromoindirubin-3′-oxime (BIO), both Wnt agonists that activate the Wnt pathway [33,34]. Scarpa et al. (2018) showed uptake of polymersome nanoparticles by hBMSCs and further intracellular delivery of BIO for greater spatiotemporal control of Wnt activation.

Rotherham et al. (2018) proposed a different WNT activation mechanism to the previously discussed endocytosis and delivery of small molecules. In this study, peptide conjugated magnetic nanoparticles were used for the remote activation and mechano-stimulation of Wnt signalling [35]. The peptide used in this study was UM206, a specific ligand for the Frizzled 1 and Frizzled 2 receptors, capable of activating canonical Wnt signalling. UM206 was attached to superparamagnetic iron oxide nanoparticles to form a magnetic complex-UM206-MNP, which allowed controlled stimulation by a magnetic field. Unlike the other mechanisms discussed, these nanoparticles did not enter the cells. Activation by a magnetic field resulted in frizzled clustering, β-catenin translocation, TCF/LEF responsive transcription activation and increased collagen and osteocalcin production and mineralisation in hBMSCs.

In summary, from the above-discussed studies, the widely accepted mechanism for activation of the Wnt pathway by nanoparticles was through internalisation, likely via clathrin-dependent endocytosis, which has been linked to the Wnt pathway. This results in accumulation of non-phosphorylated β-catenin in the cytoplasm, translocation to the nucleus and activation of osteogenic transcription factors such as RUNX2, OCN and ALP. The size of the nanoparticle appears to play a role in the magnitude of the

osteogenic effect, with smaller nanoparticles (<10 nm) eliciting a lower effect. Interestingly however, most of the studies reviewed only observed such a phenomenon under osteogenic conditions, indicating the need for activation of multiple signalling pathways for full osteogenic differentiation and the role Wnt signalling has in accelerating this process. Although the direct effect of nanoparticles on Wnt activation have been investigated, none of the studies assessed whether metal ions could potentially be released as a result of nanoparticle degradation, activating the Wnt pathway as a secondary mechanism. This would be of interest as metal ions, such as magnesium, have been shown to activate the Wnt/β-catenin pathway [36] and encourage mineralisation in hBMSCs, whilst heavy metal ions, such as lead, have been shown to inhibit Wnt activation and osteoblast activity [37]. The release of ions from iron oxide and gold nanoparticles could be investigated using potentiometers or techniques such as electrochemical impedance spectroscopy.

Additional novel techniques were also presented to further activate Wnt signalling through the use of near infrared light or magnetic stimulation. Other studies used nanoparticles, (e.g. iron oxide nanoparticles, polymersome) as delivery vehicles for sensitive ions, small molecules, or ligands (e.g. lithium or silicon).

Although much has been elucidated with regards to the activation of Wnt signalling through nanoparticles, several key questions still remain unanswered in this area of research. Changing the size and shape of nanoparticles whilst maintain the same composition and investigating the level of Wnt activation will help answer which nanoparticle geometries are best suited for bone regeneration purposes. Additionally, elucidating the signalling crosstalk between clarthrin-mediated endocytosis and Wnt signalling will further help develop more effective nanomaterials that exploit this mechanism. Finally, the positive effects of nanoparticles on Wnt activation have been predominantly demonstrated under osteogenic culture conditions only. Elucidating why Wnt pathways are activated under such conditions and not under basal culture conditions, will help develop nanotechnologies that are able to encourage bone regeneration for other bone diseases, such as osteoporosis.

3.2. Nanotopography

Fourteen publications in the review discussed the mechanism of activation of Wnt signalling by nanotopographies. With nanoparticles, the activation of the Wnt pathway was predominantly through the canonical Wnt/β-catenin pathway. Research on nanotopographies have demonstrated similar findings with the majority of the studies also demonstrating activation through the Wnt/β-catenin pathway, however alternative activation through non-canonical pathways, such as the planar cell polarity pathway (PCP) or the Wnt/calcium (Wnt/Ca^{2+}) pathway have also been proposed.

Fu et al. (2020) demonstrated that nanotopographies activated the Wnt/β-catenin pathway using murine mesenchymal stem cells (MSCs) and murine pre-osteoblastic cells (MC3T3-E1) [38]. The study showed ordered-micro and disordered-nanotopographies could modulate cells through contact guidance (Fig 4). The micropatterns guided cell orientation, whilst the nanopatterns were thought to assist in cell spreading and osteogenic differentiation. The titanium surface with an ordered micro-structure and a disordered nano-branch topography showed greater surface hydrophilicity, increased cell adhesion, cell orientation and spreading. These topographical changes were thought to modulate cell shape and nuclear orientation, resulting in an increased expression of α5β1 integrins, cadherins, β-catenin and osteogenic genes such as *RUNX2, ALP, OCN* and *OPN*. In the presence of Dickkopf1 (Wnt/β-catenin signalling antagonist), there was a decrease in the expression of osteogenic genes, highlighting the role of the Wnt/β-catenin pathway in nanotopography-induced osteogenic differentiation. The authors also confirmed the findings *in vivo* by demonstrating improved bone formation around the micro/nanopatterned implants in male Sprague Dawley rat tibias. Fu et al. (2020) proposed that when cells contact the micro and nanopatterned surfaces, integrin α_5 and integrin β_1 on the cell membrane are stimulated, leading to clustering of integrins and the formation of focal adhesion complexes. Although the crosstalk between focal adhesion kinase (FAK) and Wnt signalling is still being fully elucidated, a study by Saidak et al. (2015) demonstrated that peptide activation of α5β1 integrin leads to PI3K/Akt activity, which negatively regulates GSK-3β activity and can lead to

accumulation of cytosolic β-catenin [39]. This would lead to β-catenin nuclear translocation, interaction with TCF/LEF and upregulation of transcription factors such as RUNX2, OCN, ALP and OPN for osteogenic differentiation. Sun et al. (2016) demonstrated that BMSCs from FAK knockout mice express lower levels of mRNA for Wnt ligands (Wnt1, Wnt3a, Wnt10b) than BMSCs from control mice. FAK knockout mice also had lower levels of phosphorylated GSK-3β than control mice indicating two modes by which FAK can crosstalk with Wnt signalling [40].

Zhou et al. (2018) proposed a similar mechanism using strontium doped hydroxyapatite (Sr-HA) nanorods on microporous titania (TiO_2). The *in vitro* study showed increased osteogenic differentiation of rabbit mesenchymal cells cultured on 70nm diameter nanorods (MNR-D70) [41]. The results demonstrated increased focal adhesions through vinculin immunostaining of rabbit mesenchymal cells on MNR-D70 surfaces (Fig 5) and increased β-catenin expression. *In vivo* this translated to enhanced bone regeneration around MNR-D70 femoral implants in a New Zealand rabbit model. Yu et al. (2018b) also supported this hypothesis and showed increased α5β1 integrin expression of rat osteoblast cells on 110 nm length titanium nanotubes in an oxidative stress environment [42]. This was linked to upregulation of Wnt genes (*Wnt3a, Wnt5a, Lrp5, Lrp6, DKK1 and DKK2*) and subsequent osteogenic differentiation.

Abuna et al. (2019) and Wang et al. (2012) also highlighted the importance of Wnt in nanotopography-mediated osteogenesis [43,44]. According to Abuna et al. (2019), MC3T3-E1 cells showed improved osteogenic differentiation on titanium surfaces with nanopores of 32-40nm diameter. This effect was attenuated when the Frizzled Class Receptor 4 gene was silenced in these cells [43]. Additionally, Abuna et al. (2020) later showed that disruption of the Frizzled Class Receptor 6 (Fzd6) also suppressed MC3T3-E1 osteogenic differentiation on nanostructured surfaces [45]. Wang et al. (2012) demonstrated that titanium micro pitted/nanotubular surfaces with nanotubes of 100 nm length could enhance osteogenesis in human MG63 osteoblast cells. The study also showed an increase in the expression of Wnt3a (Wnt/β-catenin pathway agonist) and downregulation of Dkk1/2 and secreted frizzled-related protein 1/2, however the expression of Wnt5a (non-canonical Wnt pathway activator) was unaffected [44].

Chen et al. (2019) engineered titanium surfaces with ordered nanotubes and co-cultured murine osteoblasts with a murine osteocyte-like cell line (MLO-Y4) on the surfaces [46]. The osteoblasts co-cultured on 70 nm length nanotubes displayed improved osteogenic differentiation and when the surface of the nanotubes were conjugated with a sclerostin antibody (sclerostin is a negative regulator of Wnt), there was a further significant increase in osteogenic gene expression. The nanotubes provided a better topography for adhesion and spreading of osteocytes through contact guidance, and the sclerostin antibody decreased sclerostin levels in the media, preventing inhibition of the Wnt/β-catenin pathway.

Liu et al. (2019) demonstrated that lithium-incorporated nanoporous coatings on a magnesium alloy (AZ91) enhanced osteogenic differentiation of rBMSCs. Activation of the Wnt pathway was observed *in vitro* and *in vivo* [47]*.* The activation mechanism was suggested to be due to the Li ions in the nanoporous coating, however integrin signalling was not explored in this study. It was proposed that Li ions released from the coating would enter the cells through ion channels, inhibiting GSK-3β from forming the β-catenin degradation complex (Axin, GSK-3β, β-catenin and APC) and therefore increasing the concentration of β-catenin in the cytoplasm. Magnesium is known to undergo biodegradation under physiological conditions. Although the effect of the magnesium biodegradation was not investigated in the study by Liu et al. (2019), it is likely that magnesium ions released through the degradation of the material may have induced an osteogenic effect as magnesium ions alone have been shown to also activate the Wnt/ β-catenin pathway [36]

Huang et al. (2019) also proposed that Li incorporated onto sandblasted, large grit, acid-etched implant surfaces improved osteogenic differentiation in bone marrow mesenchymal cells by releasing Li-ions and increasing β-catenin levels [48]. The study also showed that Li incorporation created irregular pores of 10-50 nm in diameter, which likely enhanced the adhesion and proliferation of the cells by contact guidance.

There were five *in vitro* studies that proposed the mechanism of nanotopography mediated activation of Wnt was through alternative mechanisms. Chakravorty et al.

(2014), Hou et al. (2019), Xu et al. (2019) proposed that nanotopographies activate the NFAT pathway [49–51]. The studies used primary alveolar bone-derived osteoprogenitor cells, rat bone-marrow stem cells and MC3T3-E1 pre-osteoblasts, respectively. Hou et al. (2019) demonstrated that titanium nanotubes 100 nm in length promoted activation of transient receptor potential vanilloid 4 (TRPV4), which increased calcium ion influx, causing activation of calcineurins and nuclear translocation of NFAT (Wnt/Ca^{2+} pathway) [50]. The study found NFAT signalling promoted the expression of Wnt3a to also stimulate canonical signalling. This leads to an upregulation of transcription factors, such as ALP, RUNX2, OPN and OCN, and enhanced osteogenic differentiation. The authors proposed that TRPV4 activation was due to the surface topography induced changes of the primary cilia and the cytoplasm [52]. Xu et al. (2019) showed that ultrafine-grained pure titanium, coated with hydroxyapatite hierarchical structures, such as microscale/nanoscale pores and varying geometric shapes of short column-shaped/sheet-shaped hydroxyapatite grains, activated the Wnt/Ca^{2+} pathway [51]. The highly hydrophilic surfaces were thought to cause a high polar force, which activated NFAT and PKC pathways through G-proteins. The stimulation of G-protein activated phospholipase C (PLC) caused an increase in calcium and protein kinase C (PKC), which eventually increased the expression of NFAT and Wnt5a.

Yu et al. (2018) demonstrated that micro/nanostructured titanium (MNT) could increase the osteogenic differentiation of rat primary osteoblast cells through Rho/ROCK signalling [53]. The expression levels of phosphorylated myosin phosphatase target subunit 1 (p-MYPT1) and nuclear β-catenin were evaluated on different substrates, and MNT showed increased expression of p-MYPT1 and nuclear β-catenin. Use of a ROCK inhibitor (Y27632) suppressed the osteogenic response, confirming that ROCK plays a vital role in nanotopography induced osteogenic differentiation. The proposed pathway is known as the planar cell polarity (PCP) pathway of Wnt, where ROCK activation increases actomyosin-mediated cellular tension and promotes nuclear accumulation of β-catenin. The author also proposed that there was crosstalk between the ROCK and the Wnt5a pathways. The expression levels of Wnt5a and DKK2 were evaluated to demonstrate this feedback mechanism in the absence and presence of Y27632. In the

absence of the ROCK inhibitor, there was an increase in Wnt5a, decrease in DKK2 and LRP6 and no change in Wnt3a, DKK1 and LRP5. An anti-Wnt5a antibody was then used, which decreased the expression of p-MYPT1 and nuclear β-catenin. The authors proposed that the mechanical signals from the nanostructures activate the ROCK-signalling pathway, inducing high Wnt5a expression as a feedback loop to promote osteogenesis. Such cross-talk between Wnt signaling pathways and Rho GTPases are discussed in detail in a review by Schlessinger et al. (2009) [54]. A study by Khan et al. (2019) using human bone marrow-derived multipotent stromal cells on sand blasted acid etched topographies also showed osteogenesis to coincide with surface-induced morphological changes and upregulation of Wnt5a [55].

In summary, the majority of the publications proposed that nanotopographies activate the canonical Wnt/β-catenin pathway to induce osteogenesis. In contrast to nanoparticles, here, the activation of this pathway was shown to be through stimulation of integrins and FAK, which results in elevated expression of Wnt ligands (Wnt3a) and phosphorylation of GSK-3β. Incorporation of lithium ions within the nanotopographies was shown to further increase Wnt activation. The spacings and geometry of the nanotopographies were found to play an essential role in osteogenic differentiation by affecting cytoskeletal reorganisation, even under oxidative stress, which is promising for improving treatments such as osteoporosis. The micro/nanostructures were also shown to activate non-canonical pathways through the PCP or Wnt/Ca^{2+} pathways and through crosstalk between ROCK and Wnt5a.

Although several mechanisms for activation of the Wnt pathway have been proposed, none of the studies Investigated how nanotopographies may directly influence the adsorption of Wnt ligands. Nanotopographies are known to affect the hydrophobicity of surfaces and subsequently protein adsorption. Many studies have investigated the effects of nanotopographies on the attachment of serum proteins, such as fibronectin and albumin, [56–61] however no studies have yet emerged investigating how nanotopographies influence attachment of Wnt ligands. This may offer a further mechanism for how nanotopographies may modulate Wnt signalling.

Similar to the nanoparticles, the true effect of geometry on Wnt activation by nanotopographies is yet to be fully elucidated. A study investigating the effect of altering nanotopography parameters, such as width, height, spacing and curvature, on the activation of Wnt would help answer which parameters are key to encouraging Wnt activation and subsequently bone regeneration. Such information will be critical for developing more effective osteogenic implant surfaces. With the emergence of advanced manufacturing methods, such as ultra-short pulsed laser ablation, there is exciting potential to modify such parameters in a systematic manner to answer this fundamental question. Additionally, several different Wnt pathways were identified to be activated by nanotopographies. Blocking individual pathways and assessing the impact on bone formation will help elucidate which pathways are most important to target for bone regeneration.

Nevertheless, it is clear that Wnt pathways can be mechanically stimulated without the need for the release of ions or other potentially cytotoxic agents. The topographies guided the cells towards osteogenic differentiation; thus offering a potentially safer option than ion delivery for clinical translation.

3.3. Scaffolds

Eight publications investigated how scaffolds activated Wnt signalling. The majority of scaffolds activated the Wnt pathway via release of nanoparticles or ions, such as iron oxide nanoparticles (IONP), lithium-ions (Li), lanthanum (La) and boron (B). Xia et al. (2019) demonstrated that the release of IONP from a calcium phosphate (CPC) scaffold activated the Wnt/β-catenin pathway [62]. The research involved the incorporation of powdered 9 nm diameter IONPs in a CPC scaffold at different concentrations, with 3% IONP showing enhanced osteogenic differentiation of human dental pulp stem cells (hDPSCs). The authors showed that expression of β-catenin and WNT1 increased and Dickkopf-related protein 1 (DKK1), a negative regulator of Wnt, decreased in the CPC + 3% IONP group. Treatment of the hDPSCs with IONPs alone also enhanced osteogenic

differentiation, indicating the mechanism of action was predominantly through the presence of the nanoparticles. Although the exact mechanism of enhanced Wnt signalling was not established, the authors proposed endocytosis of the IONPs to be the likely mechanism as previously described in the nanoparticle section.

Liu et al. (2018) used entangled titanium wire porous scaffolds (ETP) coated with a 4 to 6 μm layer of lithium by micro-arc oxidation (Li-MAO) in an attempt to enhance the osteogenic potential of the scaffold [63]. Lithium chloride was used as the Li source, and a concentration of 0.02 mol/L was shown to induce the greatest osteogenic response and enhance LRP-5, LRP-6, Azin 2 and β-catenin gene expression in the human MG63 osteoblast cell line. The findings were supported by enhanced bone formation in a New Zealand white rabbit bone defect model. Liu et al. (2018) proposed that the Li ions released by the nanoporous coating entered the cells via ion channels, inhibiting GSK-3β activity, subsequently increasing cytosolic β-catenin (Fig 6) and regulating osteogenic transcription factors.

Li et al. (2018) used Li-ions and proposed the exact same mechanism as Liu et al. (2018). The study used a disease model of glucocorticoid-induced osteonecrosis to determine the osteogenic effect of Li-ions. For the *in vitro* study, glucocorticoids (GC) were added to the medium, and the osteogenic differentiation of bone marrow mesenchymal stem cells (BMMSCs) were evaluated [64]. The Li-ion group with 5 mmol/L LiCl showed enhanced osteogenic differentiation even in the presence of GC. *In vivo,* the study used a Japanese white rabbit model of steroid-induced osteonecrosis of the femoral head to demonstrate the nano-lithium hydroxyapatite group had enhanced bone formation. When the scaffold was combined with erythrogenin (EPO), which induces angiogenesis, further enhancements in bone formation were demonstrated.

Hu et al. (2018) proposed that Lanthanum phosphate ($LaPO_4$) nanoparticles of 40-60 nm in diameter in a chitosan scaffold could activate Wnt signalling and induce osteogenic differentiation of rat bone marrow mesenchymal stem cells [65]. Similar to the Li-ions, the lanthanum ions released from the scaffold were proposed to enter the cells and increase the accumulation of β-catenin by inhibiting GSK3β (as demonstrated by

increased phosphorylation of GSK3β). This study also demonstrated *in vivo* efficacy, however the authors used Sprague Dawley rats with calvarial defects as their chosen model.

Gizer et al. (2020) demonstrated that a 0.9 μg/mL dose of Boron-containing nano-hydroxyapatite (B-nHAp) induced high alkaline phosphate activity in human bone marrow-derived mesenchymal stem cells (hBMSCs) after 21 days in culture [66]. Similarly, transcriptomic analysis of SaOS-2 (osteoblast cell line) treated cells showed a greater than 1.5-fold increase in genes associated with cell differentiation, TGF-β, ubiquitin proteasome, stress response and Wnt signalling pathways. The results showed an increase in Casein Kinase 1 Gamma 3 (CSNK1G3) gene expression for B-nHap only, however no change in Casein Kinase 2 Alpha 1 (CSNK2A1) and Pygopus Family PHD Finger 1 (PYGO1). Casein Kinase 1 Gamma isoforms are required for LRP5/6 phosphorylation at Thr1479 and axin binding but not necessary for Dishevelled 2 recruitment [67].

Yin et al. (2018) used Boron-containing mesoporous bioactive glass (B-MBG) to encourage osteogenesis in an ovariectomy rat model. RNA-seq of the tissues showed SET Domain Containing 7 (Setd7), a histone methylase that catalyzes H3K4me3 associated with gene transcription activation, was upregulated in the presence of Boron, as was phosphorylation of GSK-3β, and nuclear translocation of β-catenin [68]. Knockdown of Setd7 in hBMSCs inhibited osteogenic differentiation in the presence of Boron. Therefore, it was proposed that the Wnt/β-catenin signalling pathway could regulate expression of Setd7 and the level of H3K4me3 during osteogenesis.

Several articles hypothesised that alternative compounds in scaffolds could also activate Wnt signalling for bone regeneration. Xie et al. (2019) proposed that Icariin (IC), a natural flavonoid glucoside isolated from the herb *Epimedium,* released from a hydroxyapatite/alginate scaffold encouraged osteogenic differentiation of rabbit bone marrow-derived mesenchymal stem cells (rBMSCs) and demonstrated enhanced bone regeneration in a rabbit radial bone defect [69]. IC was shown to increase the presence of

Wnt3a, β-catenin and therefore act through the canonical Wnt/β-catenin pathway. This hypothesis was also supported by Fu et al. (2016) [70].

Ardeshirylajimi et al. (2018) and Hosseini et al. (2019) proposed that incorporating inorganic poly-phosphate (poly-P) in electrospun polylcaprolactone scaffolds could encourage osteogenesis [71,72]. Ardeshirylajimi et al. (2018) used human adipose-derived mesenchymal stem cells (AT-MSCs), and Hosseini et al. (2019) used human induced pluripotent stem cells (iPSCs). Ardeshirylajimi et al. (2018) demonstrated poly-P increased gene expression of β-catenin and cyclin-D1 (required for progression through the G1 phase of the cell cycle) and therefore hypothesised the osteogenic effect of poly-P to act through the Wnt/β-catenin signalling pathway.

In summary, the predominant mechanisms by which the Wnt signalling pathway was activated by scaffolds was through the release of ions and bioactive molecules. The majority of the studies demonstrated signalling via the canonical pathway whereby released ions inhibit GSK-3β and increase the accumulation of β-catenin in the cytoplasm. This activated osteogenic associated transcription of genes such as cyclin-D1, H3K4me3, RUNX2, Col1a1, OCN, ALP, and OPN. No studies on non-canonical pathways were found, bringing to question the role of PCP or Wnt/Ca^{2+} pathways in scaffold-induced osteogenesis, particularly given scaffold nano and microstructures may have also played a role in cell adhesion and differentiation as previously described. Similarly, the release of microscale particles from the degrading scaffolds, may also influence Wnt activation, similar to the nanoparticle activation mechanisms previously outlined and merits further investigation. Nevertheless, the majority of studies were able to isolate the effects predominantly to the presence of ions/bioactive molecules. Although promising, the delivery of ions, such as lithium, brings to question potential off-target effects of the therapeutics, which were not investigated in the majority of the reviewed literature. Lithium has been widely used for the treatment of mood disorders and may influence the psychological state of patients undergoing treatment. It would be critical to answer whether local delivery of lithium through scaffolds would also result in altered psychological states long-term, especially should this approach prove to be effective in bone regeneration applications. Nevertheless, the prolonged release of ions

to the treatment site may be advantageous for bone regeneration applications due to the extended length of time required to regenerate functional bone [73] and the likelihood that nanotopographic and nanoparticle effects may be masked by extracellular matrix or short-lived *in vivo*.

4. Conclusion

The Wnt pathway has a critical role in stem cell renewal, cell proliferation and differentiation. It plays an essential role in the embryological development of bone and regulates homeostasis, repair and regeneration of adult bone. Wnt signalling, however, is not specific to bone tissue and its activation promotes proliferation and renewal of other cell types, such as tumour cells. Therefore, a better understanding of the mechanisms of activation of the Wnt pathway in bone regeneration is essential. This review investigated the mechanisms by which emerging nanotechnologies (nanoparticles, nanotopographies and scaffolds) modulate Wnt signalling to enhance bone repair.

The nanoparticles activated the Wnt/β-catenin pathway for bone regeneration through internalisation of the nanoparticles. The nanoparticles were internalised via the endocytic pathway or by diffusion through the cell membrane; however, the main mode of internalisation was via clathrin-dependent endocytosis. The size, surface and shape of the nanoparticles played an essential role in the process of internalisation. Endocytosis of nanoparticles results in an increased accumulation of non-phosphorylated β-catenin in the cytoplasm, which translocates to the nucleus, upregulating osteogenic genes such as RUNX2, OCN, ALP, and OPN. The exact link between endocytosis and Wnt signalling however is yet to be fully elucidated.

The nanoparticles were also used as a delivery system to carry ions, small molecules or ligands. This mode of delivery increased the bioavailability and reduced the toxicity of the ions and molecules employed. There were novel techniques reported which used mild local heat, infra-red radiation and magnetic stimulation to activate the Wnt pathway;

however, further studies are required to standardise these techniques and elucidate the molecular pathways in more detail.

Nanotopographies activated both the canonical and non-canonical Wnt pathways. The mechanism of activation of the Wnt/β-catenin pathway was through mechanostransduction by clustering of α5β1 integrins and the formation of focal adhesion complexes. The cell surface interacted with topographical changes, and there were stimulation and clustering of α5β1 integrins, which lead to the formation of more focal adhesion complexes. These adhesion complexes caused direct mechanotransduction to the cytoplasm and nucleus. Thus, there was an increase in cytosolic β-catenin, which entered the nucleus and interacted with TCF/LEF to upregulate transcription factors such as RUNX2, OCN, ALP and OPN for osteogenic differentiation. The mechanotransduction also indirectly stimulated autocrine and paracrine secretions. This caused an increase in the expression of Wnt3a and decreased the expression of DKK1/2 and sFRP1/2. The increase in Wnt agonist further activated the frizzled receptors and Dvl to further increase signalling. This activation inhibited the GSK3β complex and increased β-catenin accumulation. Therefore, nanotopographies activated the Wnt/β-catenin pathway through direct and indirect mechanotransduction.

The non-canonical Wnt/Ca^{2+} pathway was also stimulated by activating TRPV4 receptors and increased calcium ion influx. This increased nuclear translocation of NFAT and upregulated transcription factors such as ALP, RUNX2, OPN and OCN. The PCP pathway was activated through cytoskeletal rearrangement. The geometry and spacing played an essential role in activating specific pathways and studies showed improved bone regeneration with micro-ordered and nano-disordered topographies.

There could be overactivation of signals due to the release of ions or nanoparticles; however, this is unlikely with static nanotopographies. This could, therefore, reduce the oncogenic risk associated with exogenous modulation of the Wnt signalling pathway. The geometry and spacing played an essential role in activating specific pathways, and many studies showed improved bone regeneration with micro ordered and nano

disordered topographies. The mechanisms of activation of different pathways, however, was not clearly illustrated. Further studies are required for a deeper understanding of the mechanism.

The widely accepted mechanism for the activation of the Wnt pathways by scaffolds was through the release of ions and bioactive molecule release. The released ions inhibited GSK-3β complex formation and increased the accumulation of β-catenin (canonical pathway) in the cytoplasm. This further increased the expression of transcriptions factors in the nucleus such as cyclin-D1, H3K4me3, RUNX2, Col1a1, OCN, ALP and OPN.

Scaffolds delivered ions at specific concentrations to the targeted regions for a more prolonged period. This property reduced the systemic toxicity of ions; however, their biodistribution after degradation should be considered. Additional studies on novel compounds (poly-P and icariin) demonstrated promising alternatives to lithium and boron delivery, which are associated with potential off-target effects. Though the osteogenic differentiation was promising, more studies are required to ensure their efficacy and safety.

In conclusion, this review has identified that multiple Wnt pathways are involved in osteogenesis induced by nanoparticles, nanotopographies and scaffolds. The specific shape, size, surface, mode of delivery and bioactive molecules used determines the mechanisms by which particular Wnt pathways are activated and the subsequent effect on bone regeneration. This review has also highlighted potential areas of focus for future research around the use of nanotechnology to activate Wnt signalling for bone regeneration.

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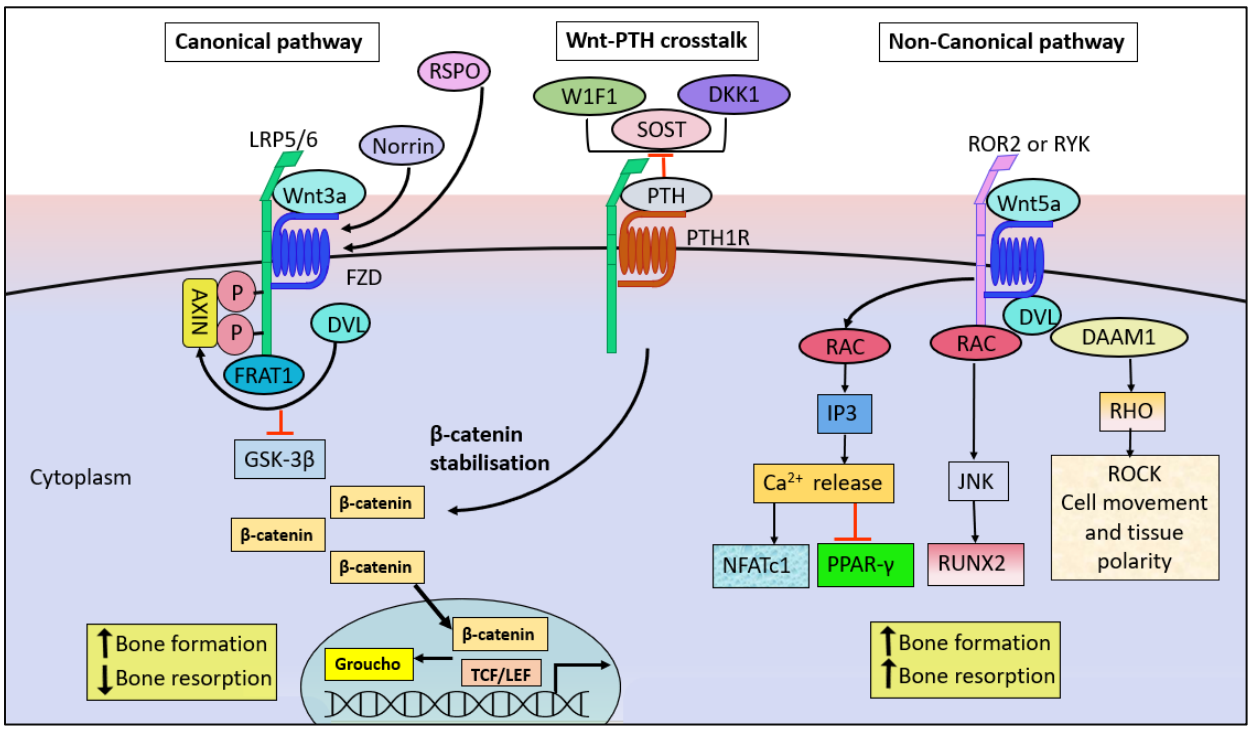

Fig 1. The canonical and non-canonical Wnt signalling pathways. In the canonical pathway (Wnt/β-catenin), Wnt3a binds to the Fzd receptor, creating a complex with LRP5/6 and Dvl. This leads to the inhibition of the GSK-3β complex allowing the accumulation of cytoplasmic β-catenin to translocate into the nucleus and bind to TCF/LEF causing downstream activation of transcription factors (increasing bone formation and preventing bone resorption). Suppression of sclerostin and Dickkopf, inhibitors of the Wnt signalling pathway, as well as β-catenin stabilisation have been linked to parathyroid hormone signalling (PTH). In the non-canonical pathway, Wnt5a binds to Fzd, with ROR as the co-receptor. This results in the activation of Dvl forming a complex with DAAM1 and downstream Rho and ROCK (PCP pathway). Alternatively, the activated Dvl forms a complex with RAC resulting in either JNK activation and RUNX2 upregulation or IP3 activation and NFAT translocation, suppressing PPARγ (Wnt/Ca2+pathway).

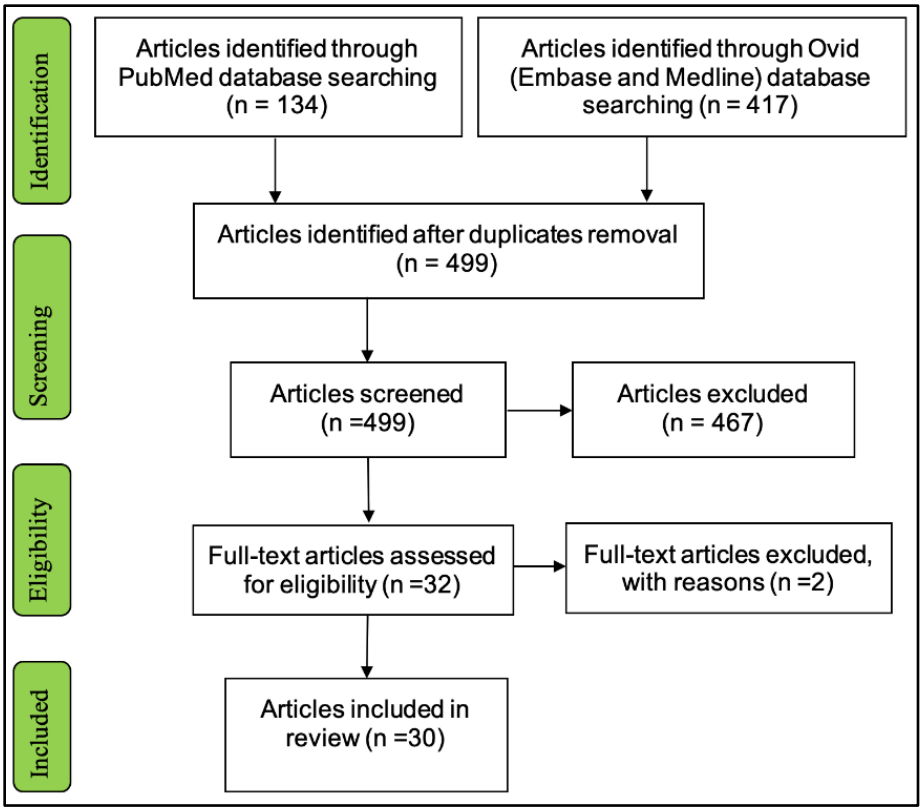

Fig 2. Flow chart demonstrating article search strategy.

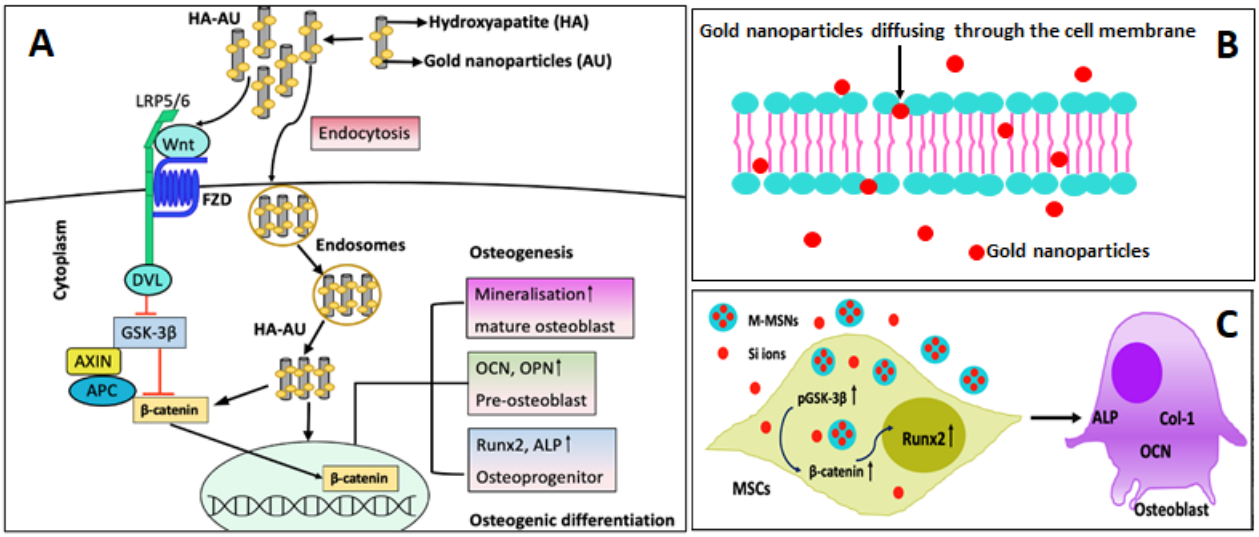

Fig 3. Proposed mechanisms of Wnt activation and internalisation of nanoparticles. **(A)** Internalisation of nanoparticles by endocytosis. **(B)** The internalisation of nanoparticles by diffusing through the cell membrane. **(C)** Activation of Wnt signalling by magnetic nanocrystals embedded in the centre of mesoporous silica shells (M-MSNs).

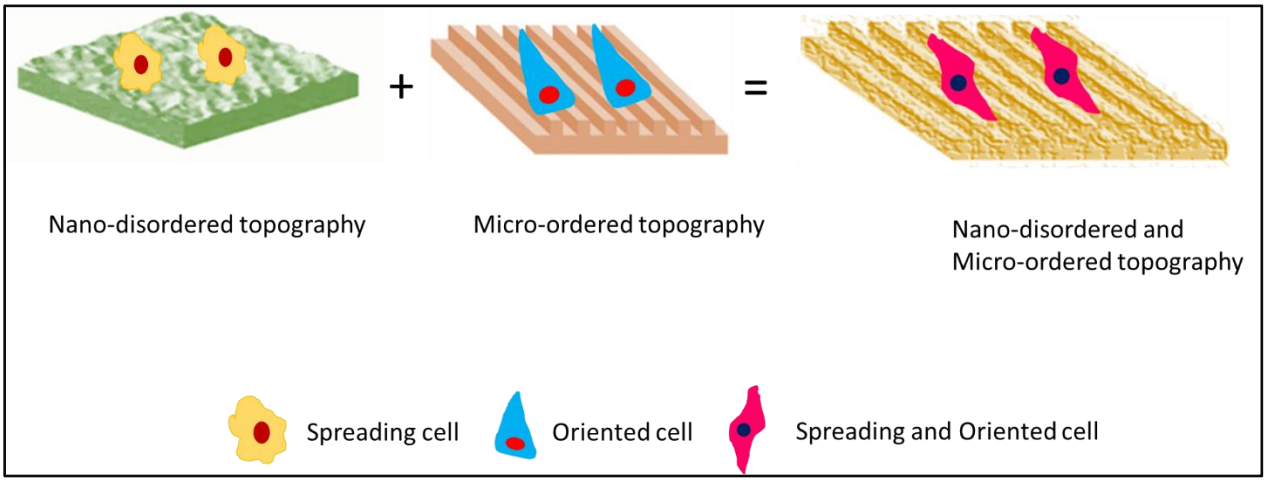

Fig 4. Cell morphology on ordered micro and disordered nanotopographies. Micropatterns are thought to guide cell orientation, whilst nanopatterns were thought to assist in cell spreading and osteogenic differentiation through integrin signalling crosstalk with Wnt signalling.

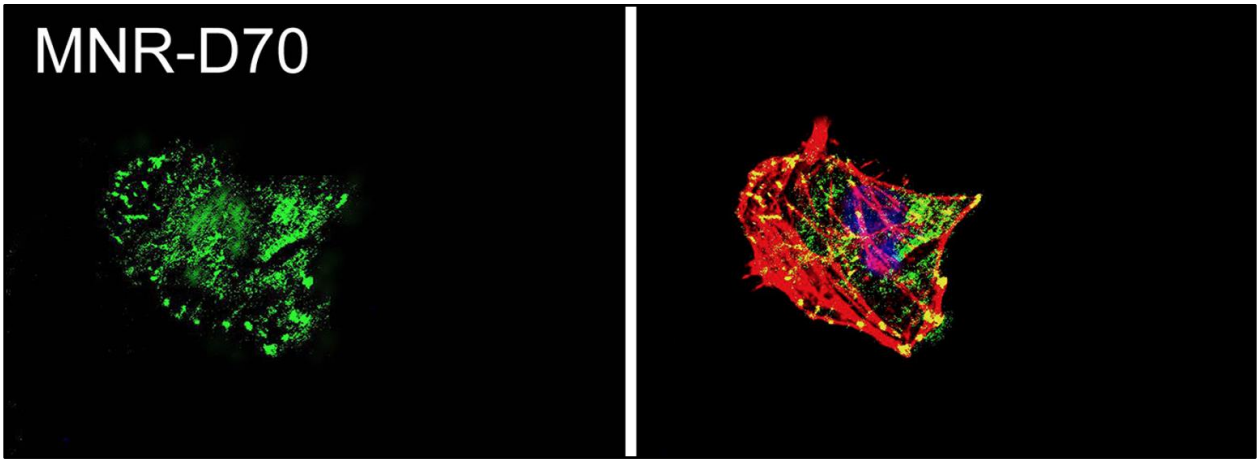

Fig 5. (A) Vinculin staining and **(B)** Tri-fluorescence (vinculin-green, actin-red, nucleus-blue) staining of rabbit mesenchymal cells on MNR-D70 showed increased focal adhesions on nanotopographies (Zhou et al., 2018).

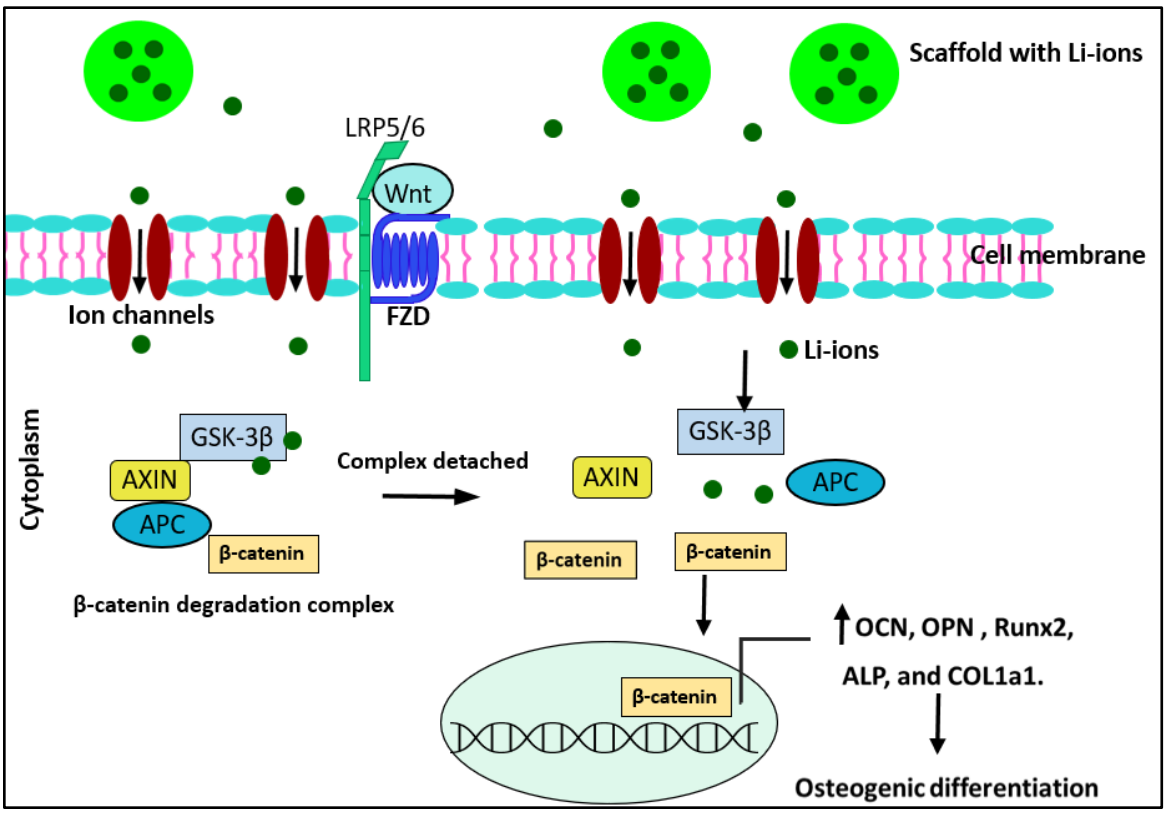

Fig 6. Mechanism of activation of the Wnt pathway by release of Li ions from scaffolds. Li ions released from scaffolds enter cells via ion channels, inhibiting GSK-3β activity and subsequently increasing cytosolic β-catenin, activating osteogenic transcription factors.