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Review Article

Bone repair and key signalling pathways for cell-based bone regenerative therapy: A review

Nur Julia N. Nasir, BSc [a], Norsyahida Arifin, PhD [b], Khairul Bariah A.A. Noordin, PhD [a] and Norhayati Yusop, PhD [a,*]

[a] *Basic and Medical Sciences Department, School of Dental Sciences, Universiti Sains Malaysia, Kubang Kerian, Malaysia.*
[b] *Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Penang, Malaysia*

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المخلص

إن التقدم في العلاج التجديدي القائم على الخلايا يخلق فرصا جديدة في علاج الاضطرابات والإصابات المرتبطة بالعظام، من خلال تحسين المرحلة التعويضية لشفاء العظام. بصرف النظر عن النهج الكلاسيكي لتطعيم العظام، اكتسب تطبيق العلاجات القائمة على الخلايا، وخاصة الخلايا الجذعية، الكثير من الاهتمام في السنوات الأخيرة. تلعب الخلايا الجذعية دورا مهما في العلاج التجديدي، نظرا لخصائصها الممتازة للتمايز إلى خلايا مكونة للعظام. يتم تنظيم تجديد العظام الجديدة من خلال مجموعة متنوعة من جزيئات الإشارات والشبكات داخل الخلايا المسؤولة عن تنسيق العمليات الخلوية. تشارك سلسلة الإشارات المنشطة بشكل كبير في بقاء الخلايا وانتشارها وموت الخلايا المبرمج والتفاعل مع البيئة المحيطة وأنواع الخلايا الأخرى داخل موقع الشفاء. على الرغم من الأدلة المتزايدة التي تم جمعها من الدراسات التي أجريت على مسارات الإشارات المرتبطة بتكوين العظام، فإن الآلية الدقيقة التي ينطوي عليها التحكم في مرحلة التمايز للخلايا المزروعة ليست مفهومة جيدا. قد يسمح تحديد المسار الرئيسي المنشط المتضمن في تجديد العظام بالتلاعب الدقيق بجزيئات الإشارة ذات الصلة داخل مجموعة الخلايا السلفية لتسريع عملية الشفاء. ستكون المعرفة المتعمقة بالآليات الجزيئية مفيدة في تحسين كفاءة الطب الشخصي والعلاج المستهدف في الطب التجديدي. في هذه المراجعة، نقدم بإيجاز نظرية آلية إصلاح العظام وهندسة أنسجة العظام تليها نظرة عامة على مسارات الإشارات ذات الصلة التي تم تحديدها لتلعب دورا مهما في العلاج التجديدي للعظام القائم على الخلايا.

الكلمات المفتاحية: العظام؛ التجديد؛ مسار الإشارات؛ العلاج القائم على الخلايا؛ الخلايا الجذعية

Abstract

Advances in cell-based regenerative therapy create new opportunities for the treatment of bone-related disorders and injuries, by improving the reparative phase of bone healing. Apart from the classical approach of bone grafting, the application of cell-based therapies, particularly stem cells (SCs), has gained a lot of attention in recent years. SCs play an important role in regenerative therapy due to their excellent ability to differentiate into bone-forming cells. Regeneration of new bone is regulated by a wide variety of signalling molecules and intracellular networks, which are responsible for coordinating cellular processes. The activated signalling cascade is significantly involved in cell survival, proliferation, apoptosis, and interaction with the microenvironment and other types of cells within the healing site. Despite the increasing evidence from studies conducted on signalling pathways associated with bone formation, the exact mechanism involved in controlling the differentiation stage of transplanted cells is not well understood. Identifying the key activated pathways involved in bone regeneration may allow for precise manipulation of the relevant signalling molecules within the progenitor cell population to accelerate the healing process. The in-depth knowledge of molecular mechanisms would be advantageous in improving the efficiency of personalised medicine and targeted therapy in regenerative medicine. In this review, we briefly introduce the theory of bone repair mechanism and bone tissue engineering followed by an overview of relevant signalling pathways that have been identified to play an important role in cell-based bone regenerative therapy.

Keywords: Bone; Cell-based therapy; Regeneration; Signalling pathway; Stem cells

* Corresponding address: School of Dental Sciences, Health Campus, Universiti Sains Malaysia, Kubang Kerian, Kelantan, Malaysia.
E-mail: norhayatiyusop@usm.my (N. Yusop)

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Introduction

Regenerative therapy for bone tissue has great potential for individuals suffering from bone defects, particularly for a bone defect that cannot heal normally or fails to recover after a long period without any sign of healing. Although the natural healing process is able to restore the defective tissue to its preinjury state, there are cases of impaired bone healing where surgical procedure and bone regeneration are required as clinical intervention.[1] The main objective of bone regenerative therapy is to restore bone function by reinstating its former properties with the newly formed bone, which will be identical to the adjacent healthy bone. The procedure involves the stimulation of new bone formation during the fracture healing period followed by continuous bone remodelling throughout adulthood.[2] Fracture repair is the most prevalent form of bone regeneration in the clinical context, where depending on the degree of injury, healing mechanisms can be different.

Bone grafting is a widely used method in clinical practice where the transplanted bone is utilised to augment bone regeneration. However, the downside of this approach includes the additional time for surgery, donor site morbidity, and limited bone availability. To address these limitations, cell-based therapy is regarded as an alternative, which allows stem cells (SCs), biomaterials, and growth factors to facilitate bone repair and regeneration.[3] Theoretically, cell therapy plays an important part in bone tissue engineering by stimulating the migration of bone progenitor cells to the injury site to promote bone repair and undergo further differentiation into bone-forming cells and tissue.[4] The process also involves the regulation of various intracellular signalling molecules with the incorporation of growth factors and cytokines that are responsible for bone regeneration.

Studies on signalling pathways in cell-based technology allow the development of a new treatment approach by targeting one or two specific pathways, which regulate the bone-forming capacity of SCs. Understanding the fundamentals of the signalling cascade, and the activated genes at the molecular level are advantageous for bone reconstruction. This paper provides an overview of the basic biology of bone repair and different modalities for regenerative therapy with the focus of reviewing the relevant signalling pathways in the osteogenesis of SCs during the bone reparative phase.

Overview of bone repair and regeneration

Notably, the bone healing mechanisms can be categorised into primary bone healing, which involves restoration of the cortex without callus formation; and secondary bone healing, which involves callus formation prior to the bone remodelling stage.[5] Primary or direct fracture healing rarely occurs since it needs a stable complex, as well as appropriate anatomical reduction of the fracture ends without any gap formation.[6] In contrast to the primary process, secondary or indirect fracture healing is more prevalent, which involves an inflammatory response of the periosteum and the neighbouring soft tissue at the fracture area.[7] The repair process also involves recapitulation of both endochondral and intramembranous bone formation aided by the homing of mesenchymal cells and osteoprogenitor cells. In secondary bone healing, the process undergoes an anabolic phase at the beginning, which then overlaps with the catabolic phase when callus volume is lessened.[7] A deeper understanding of basic bone biology is very important to formulate a specific and personalised bone reparative therapy, which effectively targets the key biomolecules within the repair site, at an optimal dose and within a precise timeframe.

To date, the bone grafting technique remains one of the most common approaches in clinical settings to repair and regenerate bone loss or defects. The method can be further classified into autograft, allograft, and xenograft. Autologous bone grafts are the gold standard in bone regeneration as it provides an osteoinductive, osteoconductive, and osteogenic potential for bone formation while maintaining the native cells' osteogenic potential.[2] In addition, it also carries minimal risk of disease transfer, lower risk of graft rejection, and lower cost of procedures.[9] However, the downside of autograft includes the limitation in graft supply, requiring additional surgery due to shape restrictions, as well as donor site morbidity.[8] Hence, the use of allograft can be beneficial, by eliminating the need for surgical procedures to harvest the graft. However, patients have increased risk of immunologic reaction and transmission of disease.[9,10] Similarly, the use of xenografts or heterologous grafts from other species may have biological limitations concerning the low osteogenic and osteoinductive capacity of the material.[11] Considering the advantages and limitations of each technique, an alternative strategy must be applied to reduce the existing risk and increase the treatment efficiency.

SC-based bone regenerative therapy

Bone tissue engineering aims to restore bone function by stimulating regeneration capacity via the use of SCs, growth factors, and scaffolds for mechanical support and transplantation.[12] The approach can either be a cell-free or cell-based procedure. Cell-free approaches refer to the utilisation of constructs to support osteogenesis, osteoconductivity, and osteoinductivity while maintaining mechanical stability such as scaffold and biomimetic materials.[13] Meanwhile, cell-based approaches focus on manipulating the differentiation of SCs or progenitor cells derived from various tissues into osteoblasts, the bone-forming cells that support new bone tissue formation.

Application of cell-based therapy to bone regenerative strategy incorporates the use of multipotent cells that are able to differentiate into osteogenic cells to restore the damaged tissues.[14] Osteoblasts are among the potential cell types that can be employed for bone repair. Other types of cells that could be used for bone regeneration are SCs, namely embryonic SCs (ESCs), induced pluripotent SCs (iPSCs), and mesenchymal SCs (MSCs).[15] Osteoblasts are

commonly employed in the bone regeneration procedure as they are naturally produced within the bone environment. Even so, they are limited in number and exhibit low proliferative capacity.[16] Although ESCs are pluripotent, the use of these cells in cell-based therapy is still controversial due to the ethical concerns associated with their primary source of isolation, and the risk of teratoma formation along with the immunologic incompatibility.[17] Hence, the use of ESCs for bone tissue engineering is not favourable. Hence, in recent years, an increasing number of studies have been dedicated to exploring the potential benefit of IPSCs in bone regenerative therapy. Nevertheless, the genetic manipulation approach used to develop iPSCs is the main concern in applying these cells to clinical applications.

Among the potential above-mentioned cells, MSCs appear more suitable for bone engineering due to their high proliferation rate and differentiation capacity, multipotency, and distinct immunological quality.[18] MSCs are capable of differentiating into bone-forming osteoblasts, chondrocytes, and myocytes in response to different conditions (transcription factors, signalling molecules, molecular pathways), which makes them good cellular candidates for bone tissue engineering.[15] Bone marrow (BM) has been the main source of MSCs since they were first isolated from this tissue in 1976.[19] However, obtaining MSCs from BM sources is considered invasive, requires general anaesthesia, and is a painful harvesting procedure. Hence, the research and clinical applications of BM-MSCs are rather limited.[20]

Nevertheless, the discovery of MSCs from noninvasive sources such as adipose tissue, dental pulp, periosteum, peripheral blood, and umbilical cord provide alternative options for the isolation of MSCs.[14,21,22] There are also a few potential sites for the isolation of MSCs within the craniofacial region. Accordingly, various types of dental SCs have been isolated, characterised, and studied such as dental pulp SCs (DPSCs),[23] SCs from exfoliated deciduous teeth (SHED),[24] periodontal ligament SCs,[25] SCs from apical papilla,[26] and dental follicle progenitor cells.[27]

Among the dental SCs, DPSCs and SHEDs are among the most studied in bone regeneration, including craniofacial bone defects. Regarding DPSCs alone, their osteogenic capacity has been frequently utilised for scaffold development to improve the osteogenic inductivity of cells embedded in scaffold.[28–30] DPSC differentiation can be induced either *in vitro* or *in vivo* by incorporating the use of several growth factors such as bone morphogenetic proteins (BMPs),[31,32] transforming growth factor beta (TGF-β),[33] vascular endothelial growth factor,[34,35] and fibroblast growth factor.[36] Similarly, SHED has also been extensively studied in the context of bone regeneration.[37–39] Although SHED is almost identical to DPSCs morphologically, which exhibit fibroblast-like cells and the ability to differentiate into osteogenic lineage, they differ in certain factors such as the expression level of growth factors.[59] Practically, with a suitable microenvironment and appropriate delivery of growth factors, osteogenic signalling molecules can be transmitted to produce a desirable result. Given the information gathered from relevant studies, it is crucial to investigate the key signalling pathways that contribute to the osteogenic differentiation of these osteoprogenitor cells, to further comprehend their characteristics for bone repair therapy.

Signalling pathways in bone regeneration

Although significant progress has been made in bone regenerative therapy in recent years, understanding the fundamentals behind the cellular and molecular mechanisms is important for successfully implementing this approach. Depending on the signals or ligands that bind to the cell-surface receptor, activation of the pathway can stimulate different downstream signalling cascades, resulting in different gene expression and SC fate. It further allows the regulation of targeted pathways in osteoblast formation. Activation of pathways that stimulate osteoblast recruitment can lead to bone formation, while stimulation of pathways involving osteoclast formation will lead to bone loss unless there is interference from other factors.[40]

Differentiation of MSCs to osteoblast progenitors in bone formation is regulated by several signalling pathways such as BMP, Hedgehog, Wnt/β-catenin, Notch, and extracellular signal-regulated kinase (ERK), as shown in Figure 1.[41,42] Table 1 depicts each of the signalling molecules' functions in bone repair and regeneration. Despite meaningful and successfully collected experimental data from both *in vitro* and *in vivo* methods, the knowledge of bone signalling pathways has not yet been applied to the current cell-based regenerative treatment.

In addition, several approaches within *in vitro* settings have been tested to study cellular signalling pathways for bone tissue engineering, comprising genomics and proteomics that can further be divided into different methodological approaches.[43] Table 2 summarises the different experimental works involving *in vitro* techniques for signalling studies in bone repair. The inhibition assay has been widely used in targeting a specific molecule in the pathway to identify the molecule's effect on cell activity, which further leads to discovering the mechanisms of the signalling pathway itself.[44–47] Typically, the inhibition assay and Western blotting are performed consecutively. Western blotting used to evaluate the protein expressed after the inhibition assay. The cytotoxic effect on the cell determined by the inhibition assay depends on the doses of inhibitor used following a specified parameters consisting of the half maximal effective concentration (EC_{50}) and the half maximal inhibitory concentration (IC_{50}), which vary for different inhibitors.[46,48] Due to the nature of the inhibitor used to repress the signalling molecules, performing an inhibition assay on the cells may affect the cell's activity such as proliferation, survival, and osteogenesis, depending on the pathway. targeted.

In the next section, the role of the relevant signalling pathways in bone formation will be elaborated upon in further detail. In studies performed between 2017 and 2022, four major pathways have been extensively studied for *in vitro* bone regeneration, namely the Wnt, phosphoinositide 3-kinase (PI3K)/AKT, ERK/mitogen-activated protein kinase (MAPK), and TGF-β pathways.

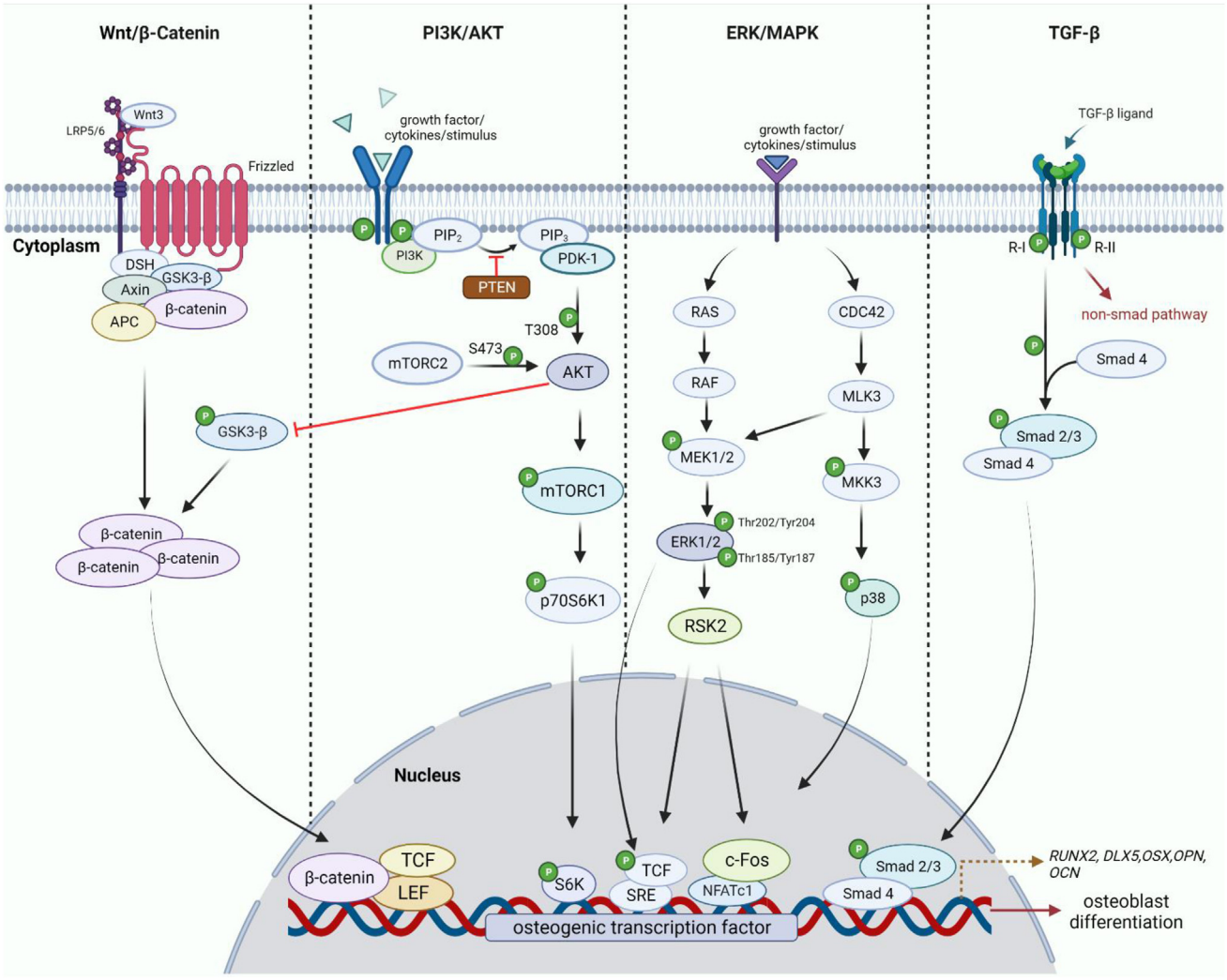

Figure 1: Schematic illustration of the identified signalling pathways for craniofacial bone regeneration. The downstream of each pathway differs depending on the ligand binding to the receptor. The activated molecule then triggers the cascade reaction of the downstream molecules which will be transported into the nucleus to stimulate the osteogenic transcription factor associated with the osteoblast formation.

Wnt pathway

The Wnt signalling pathway plays a significant role in cell regulation such as cell fate determination and proliferation during embryonic development while regulating cell maintenance, differentiation, and pluripotency in adult SCs.[49] The Wnt/β-catenin pathway comprises canonical and non-canonical branches consisting of Wnt/Ca^{2+} and Wnt/planar cells polarity (PCP) pathways.[50] The canonical Wnt signalling cascade is activated by the attachment of Wnt family proteins to the receptor. Notably, the canonical pathway is the most widely studied Wnt signalling pathway, specifically in bone homeostasis and development. The canonical Wnt pathway is associated with bone formation whereby β-catenin upregulation stimulates downstream gene expression.[51]

The role of β-catenin in the Wnt pathway includes regulating cell adhesion and mediating signalling activities, depending on the protein structural composition.[52] Wnt/β-catenin induces bone formation through β-catenin/T-cell factor 1-mediated activation of the transcription factor Runt-related transcription factor 2 (*Runx2*), which is responsible for the osteogenic lineage of MSCs.[53] The Wnt pathway plays a role in regulating osteoblast and osteoclast formation by upregulating the transcription of osteoprotegerin (OPG) while suppressing the level of receptor activator of nuclear factor kappa B ligand (RANKL), leading to a decrease in the RANKL/OPG ratio.[54] Higher OPG expression in the RANKL/OPG ratio also inhibits the differentiation of osteoclasts while promoting osteoblast differentiation.[55,56]

Theoretically, the Wnt pathway is regulated by various effectors that either act as agonists or antagonists. A study of the Wnt pathway is demonstrated by targeting the signaling molecules and further analysis of gene and protein associated with mature bone formation. Recent studies on a Wnt inhibitor, Dikkopf 2 (DKK2), which binds to low-density lipoprotein receptor-related proteins 5 and 6,

Table 1: Key signalling molecules involved in bone repair and regeneration.

Signalling molecules	Features	Function in bone regeneration	References
Extracellular messengers			
BMP-2	Osteogenic regulation factor	Involved in enhancement of ALP activity, regulation of Dlx5 in osteogenic differentiation (specifically target)	68
BMP-4	Osteogenic and chondrogenic regulation factor	Increases in BMP4 expression activates the phosphorylation of Smad1/5/8 in Smad signalling. BMP4 acts as an early stimulator of endochondral ossification	69,70
BMP-6	Osteogenic regulation factor	Regulation of BMP6 via Smad1/5/9 induces osteogenic differentiation in hMSCs.	71
BMP-7	Osteoblast differentiation factor	Regulates osteoblast lineage determination by inducing Osf2/Cbfa1 expression before any other osteoblast-specific gene	72
BMP-9	Osteogenic regulation factor	Stimulates early and late osteogenic differentiation and enhances the osteogenic marker expression in MSCs. Activation of Smad1/5/8 also depends on BMP-9-induced osteogenic differentiation of MSCs.	73,74
TGF-β	Osteoblast differentiation factor; bone mineralisation regulator	Induces osteoblast differentiation, proliferation, and migration with the support of various signalling molecules such as Smad, and AKT. It also regulates mineralisation of the bone matrix.	75,76
p38MAPK	Osteoblast differentiation regulator	An important regulator of early bone formation and development. p38 bound to BMP2/7 contributes to Runx2 and Osx transcriptional activity	77,78
ERK1/2	Osteoblast differentiation factor	Regulates lineage specification into osteoblast and osteoclast differentiation	79
Intracellular messengers			
Runx2	Osteogenic transcription factor	Also known as core-binding factor subunit alpha-1 (Cbfa1), an important transcription factor that regulates the expression of major bone matrix protein genes through osteoblast-specific *cis*-acting element (OSE2), a direct binding site in the promoter of several osteoblast-specific genes.	80
Osterix (*Osx*)	Osteoblast-specific transcription factor	Also known as transcription factor Sp7. Downstream of *Runx2* and is particularly expressed in osteoblast lineage cells. *Osx* is only present in mature osteoblasts; however, it acts as a negative regulator as it inhibits osteoblast proliferation via the Wnt/β-catenin pathway.	81
Dlx5	Positive regulator of osteoblastogenesis	Promotes osteogenic differentiation in MSCs in cooperation of BMP-2	68
Osteocalcin (OCN)	Bone formation marker; bone turnover marker	Vitamin K-dependent protein that is secreted only by mature osteoblasts into the bone microenvironment, which then induce the formation of hydroxyapatite crystals. Production of OCN only occurs at the mineralisation phase of bone formation.	82,83
Alkaline phosphatase (ALP)	Bone mineralisation regulator; bone turnover marker	Resides in osteoblast membranes and is then released into circulation. It also assists in hydroxyapatite production by providing inorganic phosphate. ALP can be utilised as a biochemical parameter for osteoblast formation and rate of fracture healing.	84–86
Bone sialoprotein (Bsp)	Bone development regulator, mineralisation regulator	Produced by osteoblasts and assists in cell attachment by promoting adherence of osteoblasts and osteoclasts to the matrix. It stimulates hydroxyapatite crystal formation in bones and teeth.	87,88
Msx1	Craniofacial bone formation regulator	Involved in morphoregulation of craniofacial development as well as teeth and alveolar bone formation. Expression of *Msx1* is also interrelated with other genes that regulate osteogenic cell lines such as *Runx2*,and *BMP2*.	89,90
Msx2	Craniofacial bone formation regulator	Mainly involved in cranial bone formation. *Msx2* stimulates MSCs into osteoblast lineage along in a Runx2-independent pathway and is induced by BMPs. *Msx2* also supresses *PPARγ* and *C/EBP* transcriptional activity from progressing into adipocyte lineage.	91
β-catenin	Osteoblast differentiation factor	Its activation regulates bone-forming osteoblast activity and osteoblast lineage determination via the canonical Wnt pathway.	92

Table 2: Application of *in vitro* techniques in signalling pathway studies.

In vitro technique	Type of method	Principle	Samples Analysed	Key Findings	References
Genomics	Microarray analysis	Identification of gene expression profiles involved in osteogenic differentiation	Study of gene expression profiling microarray data during osteogenic differentiation of BM-MSCs, focusing on several signalling pathways based on KEGG pathway analysis: LPS-mediated, PI3K/AKT, and Wnt.	Microarray data from database repository revealed several upregulated differentially expressed genes (DEGs) related to the biological processes and pathways.	99
	Real-time PCR (qRT-PCR)	Assessment of expression of bone-specific markers or gene expression of the signalling molecules in the osteogenic differentiated cells	Study of the effect of the PI3K inhibitor, LY3023414, on osteogenesis and osteoclastogenesis for bone remodelling using a preosteoblast cell line and bone marrow-derived macrophage cells (BMMs).	Treatment with a PI3K inhibitor led to reduced mRNA expression of osteoblast-specific genes in the preosteoblast cell line as well as expression of AKT in the preosteoblast cell during osteoclastogenesis in the BMMs.	96
Proteomics	Specific inhibition assay	Assessment of the effect of pathway knockdown	Preosteoblast cell line MC3T3-E1 was subjected to a specific inhibitor targeting the PI3K pathway	Effect of inhibitor treatment decreased the mRNA expression of osteoblast-specific genes (*Runx2, ALP, OCN*), and expression of phosphorylated AKT related to the downstream process of the pathway.	96,100
	Western blotting	Evaluation of protein expression and signalling molecules after cells were treated with pathway inhibitors. .	MC3T3-E1 cells treated with C3G to enhance osteoblast regulation were subjected to ERK1/2 inhibition to study the involvement of the ERK/MAPK pathway in osteoblast proliferation.	Inhibition resulted in decreased phosphorylated ERK1/2 and OCN protein expression. Although Runx2 and ALP protein were not inhibited, it can be concluded that ERK was partially responsible for osteoblast differentiation due to the inhibitor effect on OCN.	101
	Immunostaining	Analysis of protein expression, distribution, and localisation of osteogenic differentiated cells	Immunofluorescence staining to examine the expression level of type I collagen in β-ecdysterone-treated MC3T3-E1 cells to study the osteogenic effect.	Expression of type I collagen in the treated group was significantly increased compared to the control group, which shows that β-ecdysterone can be implemented for bone regeneration due to its osteogenic properties.	102
	Mass spectrometry (MS)	Analysis of intercellular signalling proteins and the regulation of cellular posttranslational modifications	Comparative proteomics of gingival tissues and alveolar bone after tooth extraction to study the protein interaction and molecular mechanisms involved in periodontal bone tissue healing.	Several proteins and canonical pathways in both soft tissue and bone are interconnected for cellular organization and maintenance. This shows that both soft tissue and hard tissue are involved in bone repair and regeneration.	103

demonstrated that overexpression of DKK2 significantly affects cell viability and DNA synthesis.[56,57] Overexpression of DKK2 also results in lower mRNA levels of the osteogenic-related factors, ALP, *Runx*, and *OCN*, as well as lower protein levels of β-catenin and Wnt1.[56,57]

Meanwhile, inhibition of pathways using DKK1 and DKK2 leads to OPG expression in osteoblasts, showing that inhibiting the Wnt pathway affects the ability of SCs to produce osteogenic-related factors, which are crucial for bone formation.[58] Figure 2 depicts the mechanism of Wnt signalling in osteoblast formation. Another notable

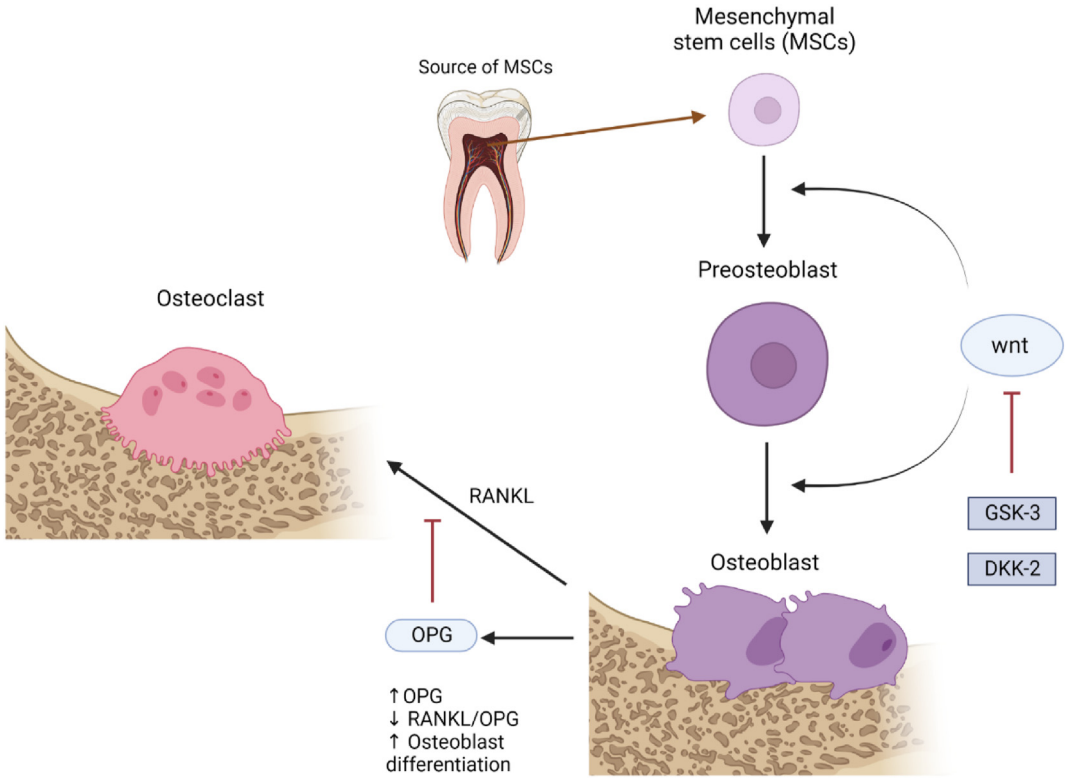

Figure 2: Schematic illustration of Wnt pathway mechanism in osteoblast differentiation. Wnt upregulates the expression of OPG in osteoblast, resulting in low RANKL/OPG ratio and inhibit the osteoclast formation which in turn increases the osteoblast differentiation.

component of the Wnt pathway, glycogen synthase kinase 3 (GSK-3), regulates various signalling pathways that modulate cellular activities including cell signalling, proliferation, and cell fate determination.[59,60] One of the GSK-3 isoforms, GSK-3β, negatively regulates the canonical Wnt/β-catenin whereby GSK-β by binding to adenomatous polyposis coli and axon, forming a protein complex that degrades β-catenin.[61] A previous study showed that GSK-3β inhibition enhanced bone formation through Runx2-dependent transcription activity.[62] Another study confirmed the role of GSK-3 in cell fate determination by demonstrating that inhibition of GSK-3β enhanced the mesenchymal progenitors with osteogenic potential by increasing the osteoblast number within 14 days.[63] Despite the great potential for activating the Wnt pathway, inhibition of GSK-3β affects cell viability if the inhibitors are used at high concentrations.[64]

PI3K/AKT pathway

PI3K has several downstream targets including phosphoinositide-dependent kinase-1 (PDK1), serine/threonine kinase AKT (also known as protein kinase B), and p70 ribosomal protein S6 kinase (p70S6K).[65] PI3K/AKT plays an important role in regulating various physiological processes such as apoptosis, proliferation, and cell differentiation. PI3K catalyses the conversion of phosphatidylinositol (4,5)-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3), which then activates PDK1 and AKT in sequence.[66] The critical role of PI3K/AKT in bone regulation has been demonstrated through the induction of progenitor cells into mature osteoblasts, as observed in AKT-knockout mice, which exhibited lesser bone mass compared to control mice.[67] PI3K inhibitors have also been utilised in studies on the PI3K/AKT pathway in order to observe changes in the bone healing process based on the expression level of bone marker genes. Inhibition of the PI3K inhibitor using LY294002 revealed diminished osteoblast differentiation in the osteoporosis group.[68] Furthermore, the inhibition of PI3K/AKT led to a decrease in genes associated with bone markers such as *ALP, OCN*, osterix (*Osx*), and *Runx2*.[68,69] Collectively, these findings suggest the potential role of the PI3K/AKT signalling pathway in bone repair and regeneration.

A recent study on the role of the PI3K/AKT pathway in bone regeneration showed that treating the samples with the LY294002 inhibitor resulted in failure of the bone tissue to fill in a gap of a fractured bone.[70] However, the study postulated possible crosstalk between PI3K/AKT and the Wnt pathway, whereby activation of AKT inhibits GSK-3β activity, leading to the accumulation of β-catenin, further confirming that PI3K/AKT regulates osteoblast function and fracture healing via β-catenin as well. This finding is consistent with another study that focused on the inhibition of pre-osteoblast apoptosis and showed that AKT activation results in the phosphorylation of GSK-3β, which leads to β-catenin activation via glucagon-like peptide-1, which is associated with bone marrow-derived mesenchymal stem/stromal cell (BMSC) osteogenic differentiation.[71,72] In addition, another study on the PI3K/AKT

pathway targeting PDK1, which is an upstream regulator of AKT, revealed that inhibition of PDK1 suppressed the ability of BMSCs to differentiate into osteoblasts *in vitro*.[73] PDK1 is partly responsible for AKT activation by phosphorylating AKT at T308 upon binding to PIP3.[74] The inhibition of PDK1 affects BMSCs at both the mRNA and protein levels, causing the expression of PDK-1 and AKT to steadily decrease over time.

Meanwhile, the mammalian target of rapamycin complex 2 (mTORC2) is responsible for phosphorylating AKT at S473, leading to complete activation of AKT, and thus being indirectly involved in regulating osteoblast differentiation. Deletion of the mTORC2 component, Rictor, leads to a lower level of ALP activity as well as the expression of the other osteoblast markers, further suggesting that mTORC2 enhances osteoblast differentiation via the PI3K/AKT pathway.[75,76] In addition, mTORC2-activated hypoxia-inducible factor 1 alpha (HIF-1) also induces BMSC to differentiate into osteoblasts. Another multiprotein complex of mTOR which is mTORC1 also involves in the regulation of protein synthesis in the cell through p70S6K1.[77] Activation of mTORC1 modulates osteoblast differentiation by regulating Runx2 expression through augmentation of *Runx2* enhancer activity.[78] The only difference between mTORC1 and mTORC2 is the complex component where mTORC1 consists of Raptor and is sensitive to rapamycin, whereas mTORC2 comprises Rictor which is insensitive to rapamycin.

Phosphatase and tensin homolog (PTEN) is a natural inhibitor of the PI3K/AKT pathway that antagonizes the action of PI3K by dephosphorylating PIP3 to generate PIP2.[79] Hence, the inactivation of PTEN may directly contribute to the activation of AKT signalling. Targeted PTEN inactivation has been investigated in several studies to understand the role of the PI3K/AKT pathway in bone formation.[80–82] The suppression of PTEN leads to a higher expression level of osteogenic markers, including OCN, ALP, and Runx2, after treatment with a specific PTEN inhibitor.[83] Treatment of BMSCs with a PTEN inhibitor further significantly enhances the osteogenic activity through the PI3K/AKT pathway, which proves that inhibition of this specific signalling component increases osteoblast cell proliferation.[84,85]

ERK/MAPK pathway

MAPK is a protein kinase family responsible for the activation of bone signalling pathways when stimulated by extracellular messengers, such as growth factors and cytokines. The three main pathways in mammalian cells are: ERK1/2, c-Jun N-terminal kinases (JNK 1–3), and p38 MAPKs (α, β, δ, and γ).[86] Each pathway class responds to different extracellular stimuli. For instance, ERK1/2 is stimulated by growth factors, hormones, and proinflammatory stimuli; whereas JNK and p38 are activated by environmental stresses and proinflammatory mediators.[87] Both ERK isoforms, ERK1 (MAPK3) and ERK2 (MAPK1), are involved in the differentiation of MSCs to osteoblast-lineage cells where activation of the pathway is activated via phosphorylation at Thr202/Tyr204 and Thr185/Tyr187 by MAPK kinase (MEK1) and MEK2.[88] Cascade activation is initiated by the binding of ligand to a membrane receptor, followed by RAS activation, which further stimulates RAF protein kinases.[88] Runx2 is an ERK substrate that is involved in osteoblast differentiation. Activation of ERK contributes to the transcriptional activity of Runx2 at the phosphorylation sites S301 and S319. Tandem mass spectrometry analysis of Runx2 phosphorylation revealed S319 to be a direct ERK substrate.[89]

Besides Runx2, ERK also has other substrates that modulate osteogenesis such as RSK2, which phosphorylates activating transcription factor 4 (ATF4) and plays an important role in late osteoblast differentiation as it regulates bone mineralisation.[90,91] However, ATF4 deficiency does not alter *Runx2* although it does reduce *Osx* expression as *Osx* is a downstream gene of *Runx2*[92]. ATF4 and *Runx2* are co-expressed in osteoblast differentiation to enhance the activation of several osteoblast-specific genes such as *Osx* and *OCN*.[92–94] Another relevant substrate of ERK that is associated with osteoblast formation is the Fos family of subunits of the AP-1 transcriptional complex, comprising FOS, FOSL1, FOSL2, and FOSB.[95] The mechanism of Fos and AP-1 stimulation in osteoblast formation by ERK activation remains unclear; however, FOS proteins via cGMP-mediated ERK are reportedly involved in bone formation by mechanical stimulation.[95,96]

The role of ERK in the osteogenic differentiation of SCs is demonstrated by increases in the phosphorylation of ERK1/2 and Runx2 during cell growth in osteogenic conditions.[97] ERK/MAPK involvement in osteoblast formation has also been demonstrated via knockout of osteoblast-specific MEK 1/2, leading to decreases in the transcriptional activities of Runx2, β-catenin, and ATF4, which that are crucial for the proliferation and survival of osteoblasts.[88,92] Also, inhibition of ERK1/2 by PD98059 specifically suppresses upstream activation of ERK1/2 which is MEK, affecting the osteogenic capacity of dental SCs by decreasing the mRNA expression levels of osteogenesis-associated genes such as *ALP*, *OPN*, *Col-1,* and *Bsp*.[98] Treatment of SCs with the selective inhibitor ERK1/2 kinase, U0126, also led to decreases in Runx2 protein level as well as the mRNA expression level of *ALP*.[99,100] Although U0126 significantly suppressed the activation of ERK1/2, the inhibitor somehow did not negatively affect the cell viability depending on the dose applied.

By contrast, data from other studies have demonstrated that inhibition of the ERK pathway suggests possible crosstalk either within the MAPK pathway or with another pathway.[101,102] Inhibition of ERK with U0126 in osteogenic-induced BMSCs resulted in no significant increase in *Runx2* gene expression, but a noticeable upregulation occurred in the protein expression of ALP, OPN, and OCN.[102] The crosstalk concept suggested that inhibition of ERK promotes p38 activity, which then modulates ALP expression and mineralisation. Thus, inhibition of the ERK pathway indicates mechanotransduction of the pathway by improvement in bone mineralisation and higher deposition of minerals into the collagenous matrix.[103]

Similarly, genetically deleted MEK1/2 in mature osteoblasts turned out to modulate bone formation by stimulating osteogenesis and the osteogenic factors, suggesting the involvement of another signalling pathway, specifically

PI3K/AKT via mTORC2 molecules.[101] Knockdown of MEK1/2 exhibits an increase in phosphorylation of mTORC2 downstream molecules, except mTORC1, which showed no changes in the downstream molecules. Therefore, it can be concluded that ERK may have a dual role in osteoblast differentiation and bone formation depending on various factors that remain unclear.

TGF-β pathway

The TGF-β superfamily consists of multiple subfamilies that are involved in growth factor secretion as regulatory peptides that control various cellular processes including bone resorption and formation. The known TGF-β subfamilies are TGF-β, BMPs, growth and differentiation factors, nodal, and activin. There are three isoforms of the TGF-β family in humans, namely TGF-β1, TGF-β2, and TGF-β3, which are located in different locations on various chromosomes.[104] In the context of bone formation, TGF-β1 is the predominant growth factor in the bone matrix among the other TGF-β isoforms. Activation of TGF-β involves the binding of the ligands to the type II receptor (TGF-βr2) which recruits TGF-βr1 activation once phosphorylated.[105] Hence, inducing signals through different TGF-β signalling can be divided into canonical and non-canonical pathways. The canonical TGF-β signalling pathway consists of TGF-β/activin/nodal activation, which requires the phosphorylation of Smad2/3 complexes joined with Smad4 followed by translocation into the nucleus where an additional co-factor is recruited to regulate target gene expression.[106] The canonical pathway via Smad2/3 activation involves binding of the ligand to the type II activin receptor, which then recruits the type I receptor, activin receptor-like kinase (ALK4/5/7).[107–109] Another canonical TGF-β pathway comprises the phosphorylation of Smad 1/5/8, whereby BMP signals are transduced by type I receptors consisting of ALK2/3/6, along with type II receptors.[110]

The BMPs involved in osteogenesis induction are BMP-2,4,6,7, and 9. In most cell types, TGF-β signals via TGF-βrII and ALK5. ALK5 induces cells into the osteoblast lineage by increasing the expression level of phosphorylated Smad2/3, which slowly decreases with osteoblast maturation.[108] One study showed ALK5 inhibition by SB431542, which targets the activin/BMP/TGF-β pathway, resulting in the decreased expression of *Runx2*, *RANKL,* and *OCN*.[111] Conversely, several other studies showed osteoblast induction when ALK5 was inhibited, leading to the elevated mRNA expression of osteoblast-specific markers such as *Runx2*, *ALP, Osx,* and *OCN* but decreased expression of phosphorylated Smad2/3.[112,113] It is postulated that the crosstalk with various pathways such as ERK, p38, and BMP result in osteoblast differentiation even when TGF-β/Smad is inhibited.[112,114]

Meanwhile, non-canonical TGF-β signalling is stimulated independently of Smad2/3 activation. For instance, TGF-β1 via PI3K/AKT/mTOR/S6K1 induces osteogenic differentiation in human osteoblasts cell lines upon treatment with a PI3K/AKT inhibitor, leading to a reduction in ALP activity.[115] The constitutively active AKT induces osteoblast induction when osteoblasts are inhibited by TGF-β1 treatment in the early stages when it exhibits increased OCN and ALP expression.[116] The study also suggested that AKT plays a significant role in the TGF-β1-induced osteoblastic differentiation of pre-osteoblast MC3T3-E1 cells. Furthermore, the activation of TGF-β via p38 MAPK regulates osteoblast differentiation by upregulating the expression of Smad1/5/8, MKK3, p38 and several other osteoblast-specific markers such as *Runx2*, *Osx*, and *OPN*.[117] The mechanism of p38 MAPK activation is suggested via phosphorylation at Thr and Tyr residue, which is stimulated by cytokines and *in vivo* environmental stress. Depending on the condition, TGF-β may also have dual roles where it is capable of regulating osteoblast differentiation in the early stages, but acts as an inhibitor in the late stages.[118–120] In the late stages of osteoblast differentiation, TGF-β inhibits the expression of Runx2 and the terminal differentiation of osteoblasts, most likely via Smad3 that suppresses matrix mineralisation.[121,122] Overall, it can be concluded that the TGF-β pathway plays a significant role in regulating molecular and cellular biological processes at the bone repair site, depending on the stage of bone cell formation.

Conclusion

Rapid development in SC research indicates huge potential for the utilisation of cells in regenerative medicine. Cell-based bone regenerative therapy serves as a great opportunity for the advancement of techniques used in bone repair and regeneration. This review emphasises the extreme necessity of understanding basic bone biology to identify the best strategies for a therapeutic approach. It is important for studies on bone tissue engineering, including biomaterials design and scaffold development, to understand the fundamental knowledge on cell growth and bone formation at the cellular and molecular levels to correctly assess the cell's responses. Apart from manipulating the external source, it is rather interesting to venture into manipulating the key players in bone formation itself, namely the bone progenitor cells. As we briefly discussed, the multipotent capacity of SCs to differentiate into various lineages offers vast opportunities for researchers to control the cell's commitment to osteoblast lineage, which is a crucial element for bone repair and regeneration. Despite the sufficient number of studies investigating the relevant pathways in bone regeneration, the lack of unanimous evidence on targeting a specific, identifiable predominant pathway remains a challenge. However, some of the studies reviewed within this paper demonstrated consistent beneficial outcomes for future therapy. Some of the reviewed signalling pathways should be explored in further detail, within *in vitro* and *in vivo* settings to obtain a wider picture of the different expression levels of osteoblastic markers at the gene, protein, cell, and tissue levels. The role of each signalling pathway could also be explored by targeting the downstream molecules and regulators within the signalling cascade. However, the crosstalk that occurs between different pathways and the system of the cellular activity when pathway alteration occurs needs to be further investigated. To date, only limited data exist on the efficacy of manipulating targeted signalling pathways for personalised cell-based bone regenerative treatment. The information provided in this review, coupled with an optimised

biomaterials design, may potentially accelerate the rate of recovery, and improve treatment modalities for bone repair.

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Conflict of interest

The authors have no conflicts of interest to declare.

Ethical approval

Not applicable.

Authors' contribution

NY designed the initial study, analysed the research material, and finalised the final manuscript. NJNN conducted the research, and collected, organised, and interpreted the data. NA and KBAAN proofread and critically reviewed the final draft. All authors have critically reviewed and approved the final draft and are responsible for the content and similarity index of the manuscript.

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