Title:

An ossifying landscape: Materials and growth factor strategies for osteogenic signalling and bone regeneration

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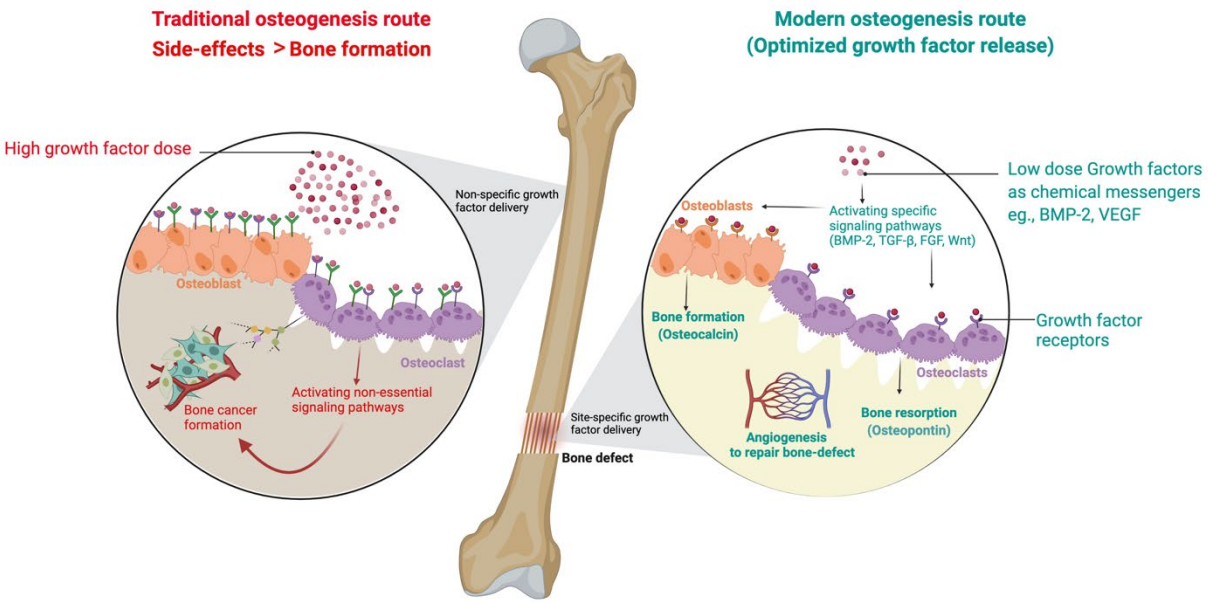
CRediT Author Statement:

Udesh Dhawan and **Hussain Jaffery:** Conceptualisation, Data Curation, Writing – Original Draft, Writing – Review & Editing, and Visualisation. **Manuel Salmeron-Sanchez** and **Matthew J Dalby**: Conceptualisation, Writing – Review & Editing, Supervision and Funding Acquisition.

Graphical Abstract



Abstract

Breakthroughs in our understanding of the complex interplay between cellular nanoenvironment and biomolecular signalling pathways are facilitating development of targeted osteogenic platforms. As critical biomolecules for osteogenesis, growth factors stimulate osteogenesis by activating key genes and transcription factors. The first half of this review presents emerging interconnectedness and recent discoveries of osteogenic signalling pathways initiating from growth factors e.g., bone morphogenetic protein 2 (BMP-2). To complement this, the second half of review proposes a number of strategies to induce osteogenesis which include metallic, organic implants, nanotopological environments as well as growth factor immobilization techniques. The drawbacks of traditional osteogenic implants and how these have been overcome by biomedical engineers in the recent years without producing side-effects have also been summarized.

Introduction

A multitude of biomechanical and biochemical stimuli emanating from the extracellular matrix in the skeletal microenvironment determine precursor cell commitment along the osteoblast lineage and bone formation. Differentiation of precursor cells entails complex regulation through an interplay of extracellular stimuli, involving extrinsic osteogenic growth factors and protein ligands (e.g., bone morphogenic proteins, BMPs; transforming growth factor-beta, TGF-β; insulin-like growth factor, IGFs; fibroblast growth factor, FGFs; and WNTs), in addition to the biomechanical force transmission through focal adhesions, gap junctions, primary cilia, ion channels, the cytoskeleton, and the nuclear envelope – all culminating in the perturbation of signal transduction pathways and gene transcription [1,2]

Networked Signalling in Osteogenesis

Recent breakthroughs in the understanding of the mechanisms of osteoblast differentiation highlight the emergence of a complex growth factor and cell signalling pathway interdependence that eludes pathway distinction (Figure 1). Commitment to the osteoblast lineage of precursors alters expression and phosphorylation of a broad network of signalling kinases within an hour, and sets of genes from disparate pathways share expression signatures [3-5].

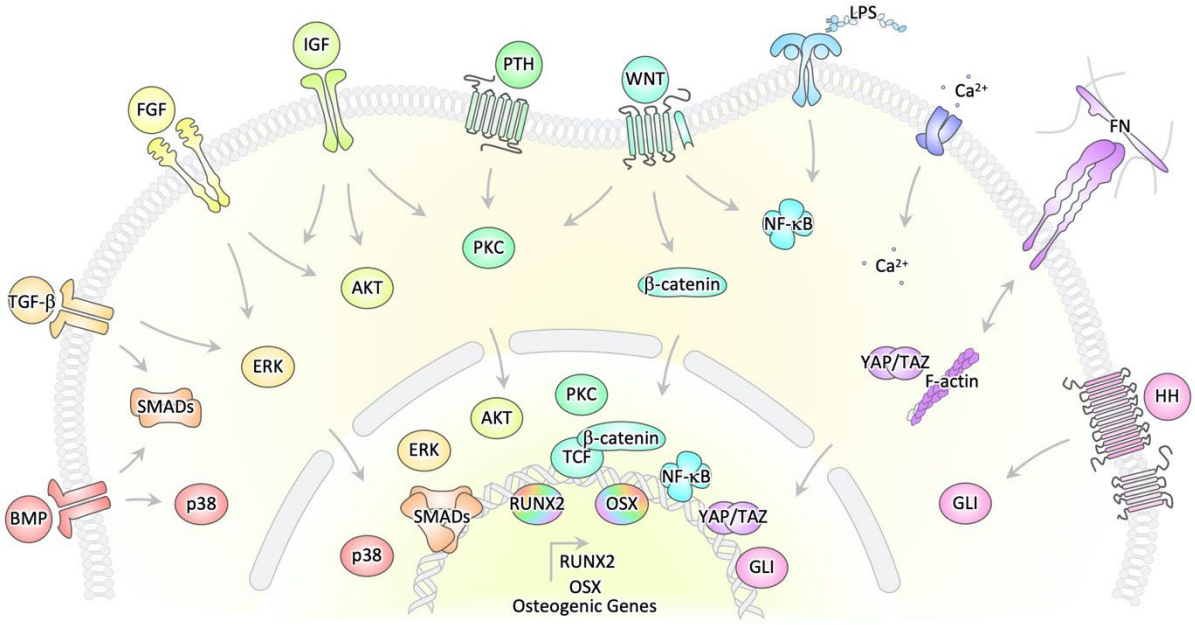


Figure 1: Osteogenesis involves a large number of cues from multiple overlapping pathways, informed by growth factors, other biochemical molecules and physicochemical stimuli in the extracellular matrix. Major osteogenic pathway ligands, and associated signal-transducing kinases or transcription factors, are highlighted. The osteoblast differentiation master regulators, RUNX2 (runt-related transcription factor 2) and OSX (osterix), primarily drive osteogenesis in coordination with other transcription factors. Selected pathway ligands include BMP (bone morphogenic protein), TGF-β (transforming growth factor beta), FGF (fibroblast growth factor), IGF (insulin-like growth factor), PTH (parathyroid hormone), WNT, LPS (lipopolysaccharide), calcium (Ca^{2+}) ions, FN (fibronectin) anchored integrin dimers, and HH (hedgehog). Key depicted downstream signal transducers include SMADs (mothers against decapentaplegic homologs), mitogen-activated protein kinases p38 and ERK (extracellular signal-regulated kinase), AKT (RAC serine/threonine-protein kinase or protein kinase B), PKC (protein kinase C), β-catenin, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), Ca^{2+} ions, yes-associated protein (YAP)/Tafazzin (TAZ), filamentous (F)-actin and GLI (glioma-associated oncogenes) transcription factors.

The prototypic osteogenic growth factors, BMPs and TGF-β, bind their specific receptors, triggering SMAD-dependent or mitogen-activated protein kinase (MAPK)-dependent gene transcription of the osteogenic runt-related transcription factor 2 (RUNX2) and osterix (OSX), and also regulate each other [6,7]. FGF and IGF binding incorporate the MAPK/ERK (extracellular signal-regulated kinases), phosphoinositide-3 kinase (PI3K)/AKT/mTOR (mechanistic target of rapamycin) and the Ca^{2+}/PKC (protein kinase C) pathways in their signal transduction [8]. A recent discovery of periodic trigger-waves of ERK signalling activity across osteoblasts showed these waves to be essential for coordinating the process of bone regeneration between osteoblasts in a spatiotemporal manner [9]. Parathyroid hormone (PTH) and PTH related peptides (PTHrPs) signal through PKC and cyclic adenosine monophosphate (cAMP)/PKA (protein kinase A) [10]. The involvement of mTOR in the osteochondral lineage is implicated as a master regulator of osteogenesis via WNT, IGF and BMPs; however, recent work has shown mTOR to additionally function directly downstream to PTH1R via salt-inducible kinase 3 (SIK3)-mediation[11,12]. WNT ligand binding can trigger β-catenin translocation, or induce Ca^{2+}/PKC and crosstalk with the master regulator of gene transcription, NF-κB (nuclear factor kappa-light-chain-enhancer of

activated B cells) [13]. NF-κB transduction can be triggered by bacterial LPS activation or by proinflammatory cytokines such as tumour necrosis factor (TNFα) or interleukin 6 (IL-6) [14]. In osteoblasts, transient NF-κB activation was found to be integral in early osteogenic lineage determination [15,16]. These well-defined signalling cascades maintain their central contribution to the canon of osteogenic extracellular factor signalling but they don't function as monoliths. Salient recent advances continue to highlight the extent of interdependence of the biochemical molecular machinery driving osteoblast differentiation and function.

Mechanosensitive Triggers of Osteogenesis

In osteogenesis, biomechanical cues are particularly important, as bone, the material osteoblasts produce, is a biomechanical tissue and a biomineral reservoir in the body. Extracellular matrix ligands, such as fibronectin (FN) bind activated integrin heterodimers, transmitting adaptor-mediated mechanical force bi-directionally between the ligands in the stiffening microenvironment and the F-actin cytoskeleton anchoring the nucleus, also triggering the Hippo pathway transcription factors, yes-associated protein (YAP)/TAZ (Tafazzin), to translocate to the nucleus for gene transcription alongside co-factors including SMADs, RUNX2, β-catenin and TEADs [17] [18]. Heterotopic ossification was recently shown to occur via a core self-amplifying, self-occurring signalling feedback loop of YAP and SHH (sonic hedgehog) signalling, in a dysregulation of the mechanosensitive machinery [19]. Hedgehog binding, localised along the cellular sensing organelle, the primary cilia, results in GLI transcription factor activation [20]. Calcium ion channels balance the extracellular mineral for ossification, while regulating intracellular Ca^{2+} signalling and have an emerging role as sensors of the microenvironment and in regulating other mechanosensitive molecules [21]. The ion channel TRPV4 (transient receptor potential cation channel subfamily V member 4), regulates cell volume expansion in response to the extracellular viscoelasticity sensed by cells, with hypo-osmotic activity coupled to increased RUNX2-driven osteogenesis [22]. Similarly, the endoplasmic reticulum Ca^{2+} leak channel TMCO1 (transmembrane and coiled-coil domain-containing protein 1) regulates osteogenesis by maintaining a CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II), RUNX2, and HDAC4 (histone deacetylase 4) signalling axis, whereby it exerts control on osteoblast activity at the epigenetic level [23]. Recent advances have proven that seemingly 'inert' stimuli including

mechanical forces and ions are dynamically linked with the biochemical and epigenetic machinery of osteoblasts.

Paracrine Osteogenic Crosstalk

Osteogenic precursors inhabit diverse cellular microenvironments wherein they interact with multiple cell types for differentiation signals. Nowhere is this more exemplary than the coordinated coupling between osteoblasts and bone-resorbing osteoclasts, which regulate each other's activity and metabolism [24]. PTH induces direct cellular osteoblast to osteoclast association, enabling SLPI (secretory leukocyte protease inhibitor) and FGF2-mediated coupling [25,26]. Coupling between osteoblasts, expressing RANKL (receptor activator of nuclear factor kappa-B ligand), and osteoclasts, expressing its receptor RANK, during bone remodeling was shown to act in reverse to previously accepted dogma – signalling into osteoblasts through RANKL-presenting extracellular vesicles – increasing osteoblast bone formation via mTOR [27].

In the bone marrow niche, peri-arteriolar stromal cells expressing the prototypical adipocyte hormone, leptin receptor (LEPR) and osteolectin were shown to transiently expand to perform osteogenic fracture repair and retain PIEZO1 Ca^{2+} ion channel-dependent mechanosensitivity [28]. PIEZO1 was also found to be integral to osteoblast coupling to osteoclasts by regulating the expression of specific collagens through YAP signalling [29]. Meanwhile, CXCL12-expressing perisinusoidal cells in the bone marrow niche were shown to be recruited for osteogenesis via activation of the canonical WNT pathway [30]. High levels of exogenous fatty acids in a vascularised microenvironment promote osteoblast differentiation by inducing fatty acid oxidation metabolism; however, simple lipid starvation can result in FOXO-driven SOX9 expression and chondrogenesis [31]. Thus, the complex microenvironments of osteoblasts determine their activity and a holistic, systems approach accounting for intra- and intercellular modes of sensing will continue to bring new mechanisms to light.

A number of strategies to activate osteogenic signalling pathways have been proposed over the last two decades [32,33]. These are generally categorized as biophysical or biochemical routes. Biophysical routes can, for example, employ flat or nanostructured cell environments to promote expression of specific integrins which later drive osteogenic signalling pathway activation. On the other hand, biochemical routes utilize growth factors as chemical messengers

to initiate specific bone regeneration-promoting signalling cascades. The mechanisms pertaining to both routes, and the benefits or drawbacks thereof, are elaborated below.

Metallic implants and osteogenesis

After implantation, biomaterials form an interface between the damaged bone and the neighboring healthy stroma including cells and tissues. The interfacial interactions play a crucial role in controlling the extent of osteogenesis, and, therefore, the choice of biomaterial that promotes osteogenesis is worthy of investigation. To date, a number of material strategies which facilitate bone formation and regeneration have been adopted. These are widely categorized as biomaterial surface modification or mechanical stimulation of cells for osteogenic guidance. Surface modification strategies can include use of element-coated implants [34], nanotopographies [35,36] or biochemical immobilization of growth factors [37]. Biomechanical forces, on the other hand, can make use of methodologies such as mechanical stimulation [38,39], and mechanical stress [40,41].

A number of studies have shown that coating of implants with elements such as niobium [42], magnesium [43], and titanium [44] promote bone formation, primarily by providing mechanical support and by enhancing the contact of damaged bone with the implant. Elemental ingestion is an alternate route of metal implant-induced osteogenesis. Shih et al. have shown that ingestion of phosphate ions from an implant surface can guide stem cell differentiation into osteoblasts through metabolic routes [45]. One of the main reasons for the use of metals for bone remodeling and osteogenesis is the comparable stiffness between metallic implants and bone tissue. However, as noted by Banerjee et al., implants with high stiffness can have a deteriorating effect on bone repair owing to stress shielding [46,47], during which stiff metallic implants impede biomechanical stimulation necessary for bone remodelling.

Nanostructured metallic platforms and osteogenesis

An alternate way of promoting osteogenesis exploits the cellular ability to sense their nanoenvironment via integrins. Rosa et al. recently showed that nanotopography can guide stem cells towards osteoblast differentiation by activating the $\alpha 1\beta 1$ signalling pathway [48]. Integrins also help the cells to adhere to their micro/nanoenvironment and we have previously identified three different integrins (ICAM1, ITGAM and ITGA1) that respond to disordered nanotopography patterns and induce osteogenesis [49]. A key finding was that disorder in nanotopography promoted higher osteogenesis than a perfectly aligned nanotopography. Additionally, we have also

shown that specific nanotopographies can activate α4, αv and β5 integrins to induce expression of osteogenic proteins [50]. Thus, specific nanotopographies may be engineered which activate specific osteogenic signalling pathways. However, cells within the *in-vivo* environment also experience mechanical forces in the form of tension, compression [51] and, therefore, activation of signalling pathways via mechanotransduction is an interesting prospect. Compression or stretching of bone results in its expansion or contraction which results in pressure gradient in interstitial fluids [52]. By using nanotopography and materials with varying stiffness as models, studies have now confirmed the involvement of YAP/TAZ and Piezo1/2 mechanosensors as osteogenic mediators [53,54]. Noteworthy, recent evidence has highlighted that YAP/TAZ play a crucial role in osteogenesis through their nuclear shuttling and cooperation with Smads and BMP-2, suggesting their ever-increasing role in osteogenesis [55]. One possible route of YAP mechanosensing could be through β_3 integrins as shown by Wang et al [56]. Thus, studying change in their biochemical expression and translocation can help engineers in identifying new osteogenic molecules and signalling pathways.

Cells interacting with the implants also experience physiological forces such as sheer stress and, therefore, these parameters must be taken into consideration before designing implant nanosurfaces. We have previously engineered an *in-vitro* platform to understand the synergistic effect of nanotopography and shear stress which can help biomedical engineers to design implant surfaces which reflect realistic physiological conditions [57]. Accordingly, a number of studies over the last decade have focused on highlighting the role of shear stress in promoting osteogenesis [58,59], suggesting that careful consideration of physiological parameters may help maximize osteogenic output.

Controlled growth factor release and nanovibrations for osteogenesis

Another approach to induce osteogenesis is by regulating the bioavailability of growth factors, and a key strategy to execute this involves their administration at low dosages. Physical encapsulation and adsorption of growth factors onto suitable substrates for osteogenic applications had been widely explored in the last decade. Although these strategies are simple to implement, they display poor release profiles as growth factors are often released instantaneously with potential off-target, systemic effects [60].

To overcome this, recent studies have resorted to use functional polymeric materials which possess growth factor binding sites and allow slow release of biomolecules [61]. One reason for success of similar studies in maximizing growth factor release for osteogenesis could be the similarity between the rate of growth factor release and rate of bone formation. A plethora of similar studies back this hypothesis. For instance, Kim et al. recently developed self-assembling hydrogels by exploiting bisphosphonate interactions with positively charged nanoclay edge sites for growth factor binding [62]. The gels retained functional BMP-2 followed by its slow release over 6 weeks which resulted in nearly 12-fold increase in bone volume as compared to the control group. In extension, we have recently engineered a poly (ethyl acrylate) (PEA)-based platform to deliver ultralow dose of BMP-2 (50 ng/mL) for bone regeneration [63,64]. By using murine models and canine patients (50 μg/mL), we have showed the bone regeneration potency of PEA-Fibronectin-BMP-2 platform. Noteworthy, the concentration of BMP-2 used was only 10% of the growth factor dose (0.5 mg/mL) typically used to repair complex dog fractures. Additionally, Freeman et al. developed a platform comprising of 3D implants loaded with vascular endothelial growth factor (VEGF) and BMP-2. Their spatiotemporal experimental model showed that a slow release of VEGF and BMP-2 from the periphery and center of the scaffold is a more potent strategy for osteogenesis than the fast release. Through their model, the researchers reported a stable temporal release of growth factor for 35 days [65]. In most studies, a slow-release profile accelerated bone defect healing and angiogenesis to a higher extent as compared to freely available growth factors at a higher concentration [65].

Recent evidence has highlighted the synergistic overlap between growth factors such as BMP-2 and mechanotransducers such as YAP/TAZ. For instance, Wei et al. recently showed that YAP/TAZ play a crucial role in mediating BMP-2-induced osteogenesis via Smad complexes [55]. Through systematic experiments, the authors showed that BMP-2-guided osteogenic differentiation does not occur on soft substrates. In contrast, stiff substrates allow activation and nuclear accumulation of YAP/TAZ which is essential for osteogenic gene activation. Thus, correct mechanical stimuli is a prerequisite for BMP-2-induced osteogenesis. An important implication of their findings is the choice of substrate for growth factor immobilization, suggesting a stiffer substrate may be the preferred choice owing to its YAP/TAZ activation-ability. Nevertheless, the contribution of YAP/TAZ in osteogenesis within soft 3D hydrogel environments in the presence of growth factors still needs to be investigated.

A further approach utilizes bacteria to present growth factors for guiding mesenchymal stem cells (MSCs) towards osteogenesis. Researchers engineered bacteria with exposed $FNIII_{7-10}$ domain which facilitated cell adhesion [66]. These bacteria were also engineered to supply a constant low dose of BMP-2 to MSCs for inducing osteogenesis, highlighting the use of non-pathogenic bacteria for stem cell engineering.

An interesting new strategy to induce osteogenesis utilizes a nanovibrational bioreactor which delivers ultra-precise vibrations to MSCs for guiding their differentiation into bone cells [16,67,68]. We have shown that stimulating cells with vibrations in the form of nano-meter amplitude results in elevated expression of osteogenic biomarkers such as osteocalcin, osteopontin, alkaline phosphatase and BMP-2. Further mechanistic experiments reveled activation of TRP-β-catenin signaling pathway in response to nanovibrations. It is likely that for major bone regeneration, (following, e.g., cancer or trauma) cell therapies will need to be used alongside load bearing, bioactive, implant materials. Approaches such as nanovibrational MSC stimulation may help prime cells to be co-implanted with advanced materials to allow major defect regeneration [69].

Some approaches to achieve osteogenesis are highlighted in Figure 2.

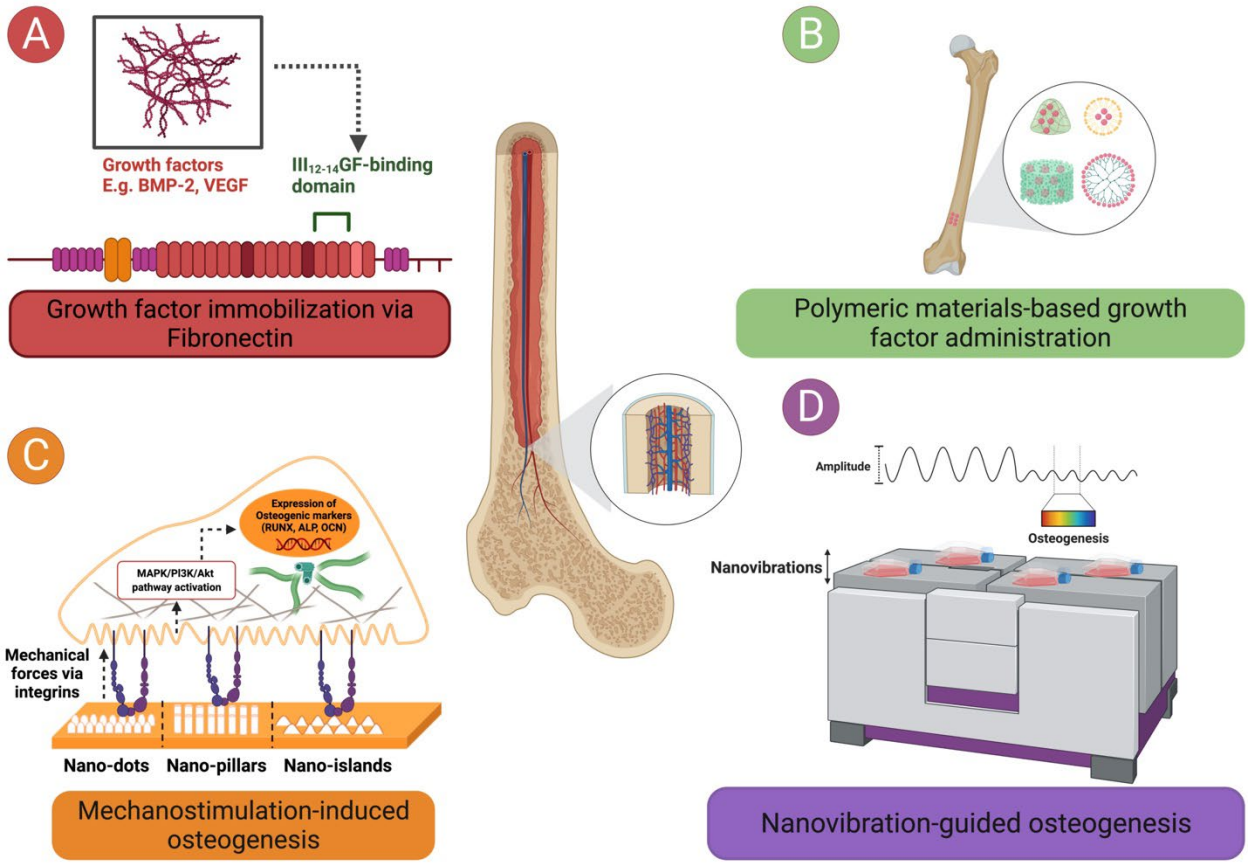


Figure 2: Schematic representation of latest strategies to induce osteogenesis. A. Exploiting the growth factor-binding domain (III_{12-14}) of Fibronectin to bind potent osteogenic growth factors such as Bone morphogenetic protein (BMP-2) and Vascular endothelial growth factor (VEGF). Using this strategy, a stable yet continuous low-dosage of growth factors can be administered to the deformed bone area which maximizes osteogenic output. **B.** Growth factors can be covalently encapsulated within micelles, hydrogels and dendrimers and stimulated release of growth factors can be achieved using physiological pH as a cue. **C.** Exploiting integrin-cell nanoenvironment interaction to activate osteogenic signalling pathways. Through this approach, specific nanotopographies can be identified that activate specific osteogenic signalling pathways. **D.** The most recent trend in inducing osteogenesis features a technique called nanokicking using a nanokick bioreactor which delivers vibrations to MSCs at a specific amplitude to guide them towards osteogenesis.

Future Considerations

It is noteworthy that irrespective of surface geometry, metallic implants can induce severe immunological responses, and recent studies have shown that their disadvantages can outweigh the benefits [70,71]. A key drawback of using metallic implants is the oxidative corrosion and their degradation-prone nature to neutrophils, generating material debris which can induce immunogenic response [72]. Neutrophils also contribute to implant degradation via hydrolytic processes. Consequently, metallic implants can induce release of cytokines such as IL-4, IL-6 and TNF-α and cause inflammation at the site of implantation (Figure 3) [73,74]. Additional drawbacks of metallic implants include high stiffness which prevents bone remodelling and additional surgical procedures for implant removal after treatment duration. Although some biomolecules such as TGF-β are involved in angiogenesis, which is an important process during bone formation, evidence also points towards its inflammatory effects [75]. Therefore, it is imperative to quantitatively evaluate its expression along with other cytokines such as Interleukin-1 (IL-1), IL-6, tumor necrosis factor α (TNF-α) wile designing biocompatible metallic implants. To overcome these challenges, metal implants can be substituted with organic and polymeric materials. This is because the physical and chemical composition of the polymeric materials can be carefully tailored to induce controlled-immune response for angiogenesis and osteogenesis [76]. Furthermore, nanotopographies can also be engineered using organic materials to enable cell-nanoenvironment interactions for activating osteogenic signalling pathways.

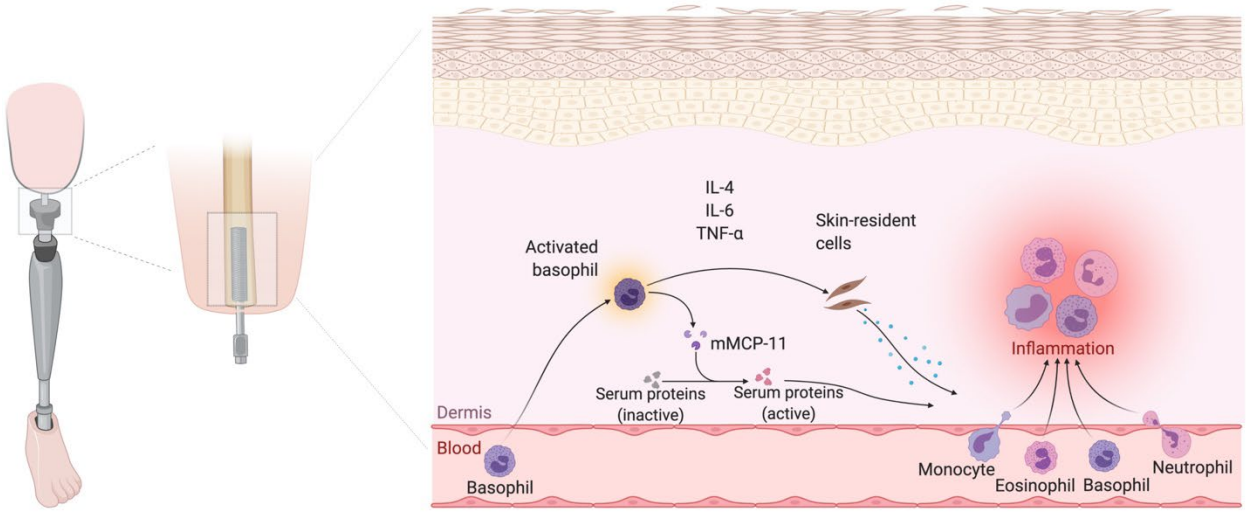


Figure 3: Schematic representation of the biomolecular events involved in the immune response following metal implantation. Foreign body reaction induced by implants can activate

basophils which then secrete IL-4 and other pro-inflammatory cytokines. Basophils can also express mouse mast cell protease 11 (mMCP-11) which is associated with skin swelling. Monocyte marker CD14 has been found to be elevated in patients with metal implants and suffering from metallosis, indicating its role in physiological response to foreign bodies. Eosinophils are associated with failure of metal-on-metal arthroplasty while neutrophils are one of the first cells that interact with implant biomaterial and are commonly associated with inflammation on Titanium implants. Collectively, the image summarizes the immune response to metallic implants.

Besides inviting an unnecessary immune response, metallic implantation requires highly invasive surgeries. Furthermore, metals are also limited in their intrinsic ability to provide a native environment for cells to proliferate. Polymeric materials on the other hand not only display a wide variety of bindings sites for extracellular matrix proteins (e.g., collagen) for proliferation and physiological functioning, but are also gaining attention due to their minimally invasive implantation methodology. Montgomery et al. recently developed (poly (octamethylene maleate (anhydride) citrate), POMAC) shape-memory scaffolds for improved cardiac function. The key feature of this scaffold was its deformation-friendly nature, allowing for implantation through a 1 mm orifice with results similar to that of open surgery [77]. Although their findings were promising, however, the material performance still needs to be validated for regeneration of hard tissue such as bone. Boutry et al. took this a step further and utilized POMAC as a strain-sensor for application in personalized orthopedic rehabilitation by assessing real-time healing [78]. Liu et al. have demonstrated the use of chemically crosslinked poly (ε-caprolactone) and exploited its shape-memory and functionalization ability to bind BMP-2 for bone regeneration [79]. These studies collectively highlight the prospect of using polymeric materials for bone regeneration.

A consistent challenge in using unmonitored growth factor dose is activation of needless cancer-related signaling pathways. For this reason, the ability of polymeric materials to bind a multitude of growth factors can be of paramount importance. By delivering ultra-low dosage and stimuli-controlled growth factor release, specific and controlled activation of osteogenic signaling pathways may be achieved with minimal side-effects.

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**Annotation: De Simone et al. remarkably visualise and model the finding of highly-defined and repeating concentric rings of activated ERK protein in Zebrafish scales. This work unequivolcally positions ERK phosphorylation as centrality to coordinating activity of entire populations of osteoblasts on a bone surface. The basic findings have implications for understanding osteogenic signalling cascades and osteoblastic tissue in broader contexts.

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**Annotation: NF-κB signalling in osteogenesis is typically described in a whole-organism manner, where systemic inflammation leads to bone loss. However, little work has been done to demonstrate what NF-κB does within osteoblasts, as part of the differentiation machinery. Mishra et al. are among an emerging set of studies showing that NF-κB, in all its complexity, is specifically regulating the cell fate of the demonstrably reversable osteo-chondro lineage in favour of osteogenesis.

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*Annotation: This work is a great exemplar of how osteogenesis is a multi-organelle and highly complex multi-tiered process. Li et al. demonstrate that the calcium leak channel TMCO1, localised to the endoplasmic reticulum, is essential to osteogenesis. It leads to the regulation of the histone deacetylase HDAC4, an epigenetic modifier, and subsequent RUNX2 activity, which controls osteoblast differentiation.

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**Annotation: This study beautifully visualises, for the first time, that osteoblasts and osteoclasts directly touch each other, aside from communicating across the extracellular milleu. Osteoblasts are demonstated to literally hold osteoclasts in an inactive state. The inclusion of parathyroid hormone perturbs the steady-state intercellular contacts in a spatio-temporal manner and leads to bone formation.

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**Annotation: Ikebuchi et al. present a landmark study, upending previous notions of osteoblast and osteoclast copupling via cytokines. Previous to this study, the central axis of osteoclastogenesis, involving inward signalling via RANK (receptor activator of nuclear factor-kappa B), was thought to function linearly - activated primarily by osteocyte-secreted RANKL. Herein, they demonstrate the backward signalling from osteoclasts to osteoblasts via intermediary extracellular vesicles expressing RANK. RANKL signalling within osteoblasts triggers osteogenesis. Whilst there are many means by which osteoblast-osteoclast coupling occurs, none may be as central for osteoclast-driven control of osteoblasts, as the mechanism described here.

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*Annotation: Matsushita et al. demonstrate that $CXCL12^{+}$ quiescent bone marrow reticular stromal cells, residing in the perivascular niche that may readily differentiate into adipocytes in steady-state, in fact become osteoblasts when there is a skeletal injury. Canonical WNT singalling coordinates this switch between adipogenic (WNT inhibited) and osteogenic (WNT activated) identities. The paper sheds light on cellular and molecular mechanisms critical skeletal regeneration and is great advancement for work concerned with bone marrow-derived stem-cell osteogenesis.

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vessels result in fatty-acid-oxidation driven ostegenic differentiation; whereas, insufficient vasculature or lipids in general results in low fatty-acid-oxidation activity and activates SOX9, causing chondrogenesis. This simple dymanic helps explain skeletal tissue phenotype in health and in repair models. There are implications for lipid-driven interventions and for adjusting dietary fatty acid sources to aid either chondrogenesis or osteogenesis.

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