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The potential roles of nanobiomaterials in distraction osteogenesis

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Abstract

Distraction osteogenesis (DO) technique is used worldwide to treat many orthopedic conditions. Although successful, one limitation of this technique is the extended period of fixators until the bone is consolidated. The application of growth factors (GFs) is one promising approach to accelerate bone regeneration during DO. Despite promising in vivo results, its use is still limited in the clinic. This is secondary to inherent limitations of these GFs. Therefore, a development of delivery systems that allow sustained sequential release is necessary. Nanoparticles and nanocomposites have prevailing properties that can overcome the limitations of the current delivery systems. In addition, their use can overcome the current challenges associated with the insufficient mechanical properties of scaffolds and suboptimal osteogenic differentiation of transplanted cells in the distraction gap. We discuss the clinical implications, and potential early applications of the nanoparticles and nanocomposites for developing new treatments to accelerate bone regeneration in DO.

From the Clinical Editor: This comprehensive review discusses the clinical implications, and potential early applications of nanoparticles and nanocomposites in the development of new treatments to accelerate bone regeneration in distraction osteogenesis.

Key words: Nanoparticles; Nanotechnology; Nanobiomaterials; Distraction osteogenesis; Bone regeneration; Bone healing; Delivery system

Nanotechnology implies the science of manipulation of single or groups of atoms at nanometeric scale.[1] The reduction of size in the biomedical materials to a nanometer scale modifies their chemical, physical and biological proprieties, resulting in new wide diversity of applications. In fact, this technology has induced a revolution in several fields of science. Although its use has shown promising results in various bone tissue engineering applications, its potential role in the context of distraction osteogenesis (DO) remains unclear. The aim of this article is to review and discuss the potential applications of nanobiomaterials to address current issues related to DO and to improve the outcome of this procedure.

Distraction osteogenesis

Distraction osteogenesis and its clinical value

Bone possesses an intrinsic capacity to heal spontaneously following injury. Nevertheless, this capacity cannot be achieved beyond a certain critical size defect and therefore an exogenous intervention is required. Several procedures are currently available to manage these large defects including the gold standard autogenous bone grafts, allografts and vascularized fibular bone grafts. In addition to the huge financial cost, these procedures have other limitations in cases of severe bone loss or when large segments of bone need to be lengthened.[2-5] DO is considered a valuable alternative in such instances. DO technique is a controlled surgical procedure that has the ability to achieve spontaneous bone regeneration by means of mechanical forces to stimulate the endogenous biological

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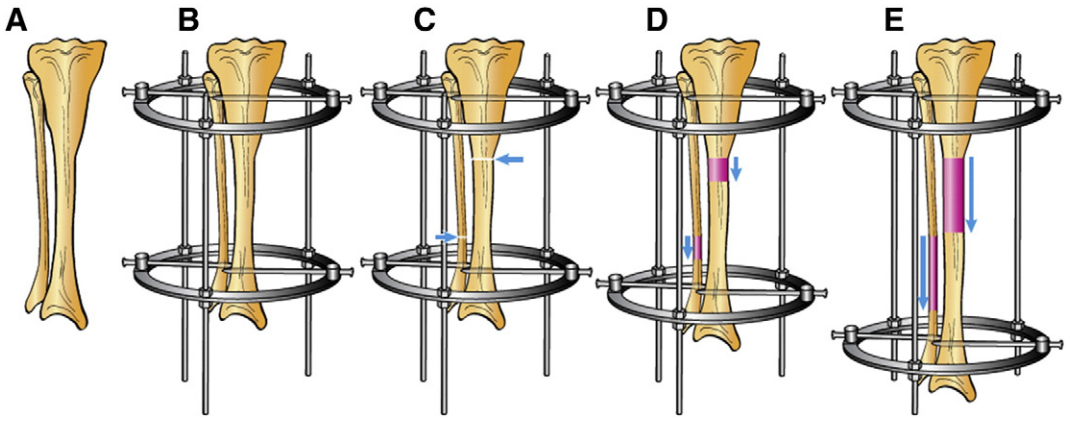

Figure 1. Description of distraction osteogenesis technique. **(A)** Showing the tibial bone that need to be lengthened. **(B)** Application of external fixator at the proximal and distal end. **(C)** Tibial and fibular osteomty. **(D)** Distraction phase. Note the new bone formation in the distraction gap **(E)** consolidation phase.

response.[6] The technique is performed as follows: the proximal and distal ends of the bone are immobilized and typically fixed by using an external fixator device followed by a low energy osteotomy to divide the bone in two segments (proximal and distal). Then, a latency phase of 5-10 days is required to allow for the hematoma formation. Subsequently, the distraction phase is initiated in which the two-bone segments are gradually distracted at specific rhythm and rate until the desired lengthening is obtained. The consolidation phase follows in which the distraction is ceased and the two-bone segments are held in place until the new bone in the distraction gap is completely consolidated. Each one centimeter of lengthening typically requires one month period of consolidation (Figure 1).[7] The external fixator can be removed once sufficient consolidation of bone is obtained.

Three modes of ossifications occur in DO. These include endochondral bone formation which dominates the early stages of DO and typically occurs external to the periosteum, intramembranous ossification which is the predominant mechanism of ossification, mainly in the late stages of DO and occurs internal to the periosteum at the proximal and distal edges of the callus. The third mode is transchondroid bone formation in which chondroid bone is formed directly by chondrocyte like cells, with gradual transition from fibrous tissue to bone.[6]

During the latency phase, immediately after osteotomy, an intense local inflammatory reaction eliciting secretion of cytokines (interleukin-1, interleukin-6), growth factors [transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), insulin growth factor (IGF) and vascular endothelial growth factor (VEGF)] and activation of Wnt signaling pathway.[6,8] This enables local deployment, differentiation and proliferation of mesenchymal cells, fibroblasts, and osteoprogenitors as well as fibrin/collagen matrix edification and capillary invasion. The osteogenic potential of these pathways is achieved by inducing the expression of bone-specific genes (e.g. Runx2, Osterix).[9] Differentiation of osteoblast is associated with an increased expression of type 1 collagen and alkaline phosphatase. Osteocalcin is also increased during matrix deposition and mineralization. Once these are achieved, a soft callus between (endosteal) and around the osteotomy bone ends (periosteal) is formed.

During distraction phase, the incipient callus is subjected to tensile stresses meant to facilitate bone regeneration in the distraction gap. The mesenchymal stem cells that migrated and proliferated into the callus differentiate initially into fibroblastlike cells. They adopt a well-defined orientation, parallel with the vector of distraction, as do their secreted collagen fibers[7] (Figure 2). As distraction progresses, the osteoblasts appear along the periosteum and in the gap area. There is increased blood flow, neovascular proliferation and ongoing up-regulation of growth factors pathways, Wnt signaling pathway and matrix proteins.[8,10] The physical forces are converted into biochemical signals which are then integrated into cellular responses via mechanotransduction. This is responsible for maintaining the dynamic balance between bone formation and bone resorption. From a mechano-modulation standpoint, the bone tissue is described as an extensively connected cellular network where the osteocytes constitute the sensory cells and the osteoblasts and the osteoclasts serve as the effector cells. Loads applied to the entire bone are related to the flow past the osteocytic processes in their canaliculi. The osteocytes can sense the flow of fluid and then produce signaling molecules that regulate osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Formation of a good regenerate that is robust enough to sustain physiological loadings requires accuracy in surgical technique, and distraction rate, rhythm and duration.[11,12]

Since its introduction by Ilizarov in early 1950s, DO technique has been utilized worldwide to treat many complex orthopedic and craniofacial conditions with satisfactory outcomes. These conditions include nonunions, congenital and acquired longitudinal bone deficiencies, and severe bone loss secondary to infections and bone tumors.[13] DO can treat large bone defects using the bone transport technique (Figure 3). In fact, the magnitude of this problem is massive as approximately 150,000 large bone defects are sustained in United States annually secondary to trauma.[14] DO is considered the best in vivo tissue engineering techniques as it has the ability to achieve spontaneous formation of de novo native bone without the need for bone grafts. In addition, DO has the unique ability to regenerate both bone and soft tissues (e.g. vessels, nerves and

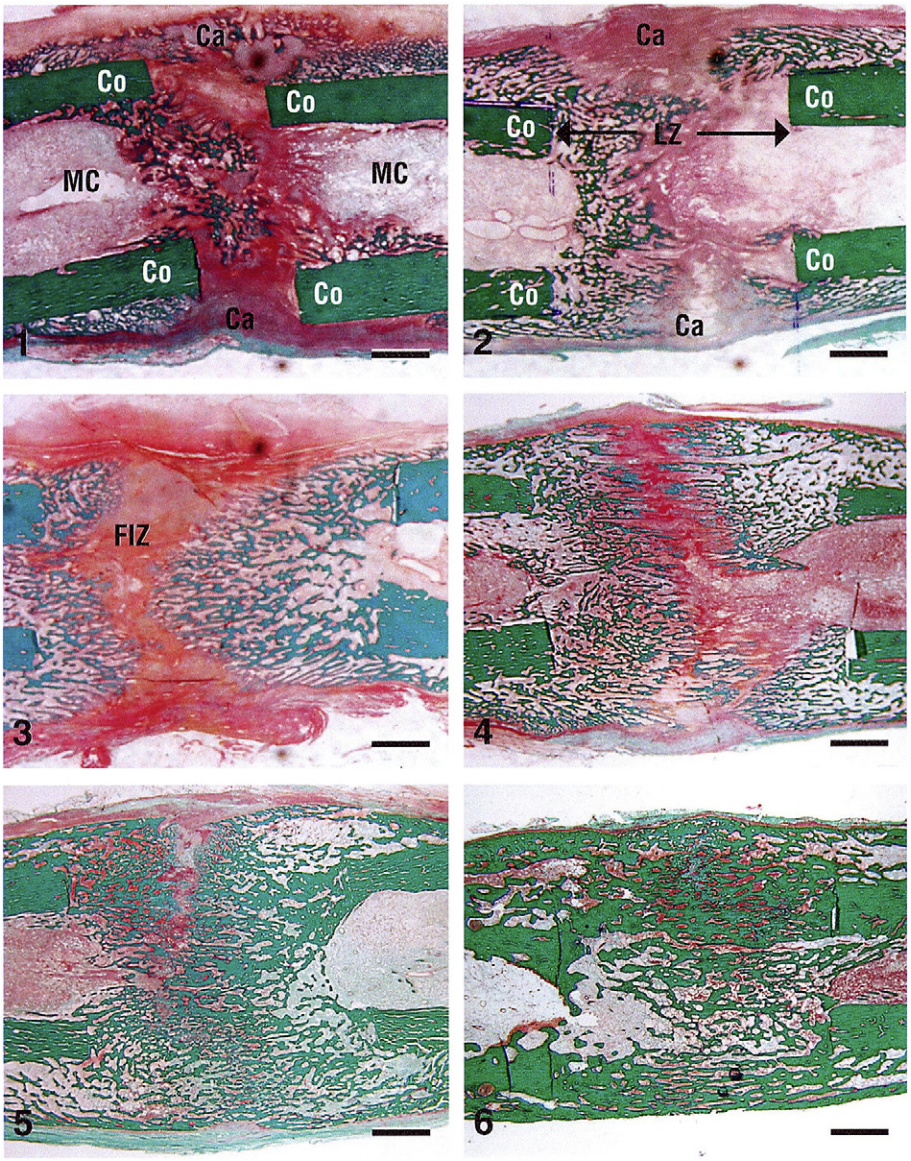

Figure 2. Histological changes (trichrome staining) during distraction osteogenesis of the tibia of 2.0 cm in a rabbit model of DO. Reprint with permission from Bone. 2000 Jun;26(6):611-7. Co: Cortex, Mc: Medullary cavity, Ca: callus, FIZ: fibrous interzone.

muscles) simultaneously. However, DO has some limitations that will be discussed in the following section.

The limitation of distraction osteogenesis and how to address it

The major limitation of DO is the long period of time that the fixator has to be left in place until the bone is completely consolidated. This consolidation period has to be further extended if a delayed or absent callus formation has complicated the course of treatment. This can be associated with unfavorable outcomes on patients such as increasing the financial burden, risk of infection, a negative psychological impact and possible subsequent surgical interventions.[13,15,16] The question then arises: can we accelerate bone regeneration in patients undergoing DO, so that the external fixator can be removed at earlier time? Several modalities have been investigated to accelerate bone regeneration during DO including the biophysical, mechanical, and biological methods (Figure 4).[10] One of these, is the exogenous application of various GFs including BMPs, VEGF, PDGF, FGFb and IGF-1 as a potential approach to accelerate bone regeneration during DO.[10] These GFs are osteoinductive due to their ability to promote the differentiation and recruitment of mesenchymal stem cells in the recipient tissues into osteoblasts that capable of forming new bone tissue.[17]

Although the exogenous application of these GFs during DO has shown promising results in the animal models of DO,[18-27] the clinical use of these GFs is still limited so far. This is mostly related to the rapid clearance of GFs, short resident time in tissues and short half-life.[28] Therefore, large doses are required in order to achieve the desired outcome. This is associated with huge cost, toxicity and unknown side effects.[29] In order to overcome these challenges, the development of a delivery system that allows sustained and sequential slow release of these GFs becomes necessary.

Among delivery systems for GFs, microparticles and particularly nanoparticles revealed very promising results for

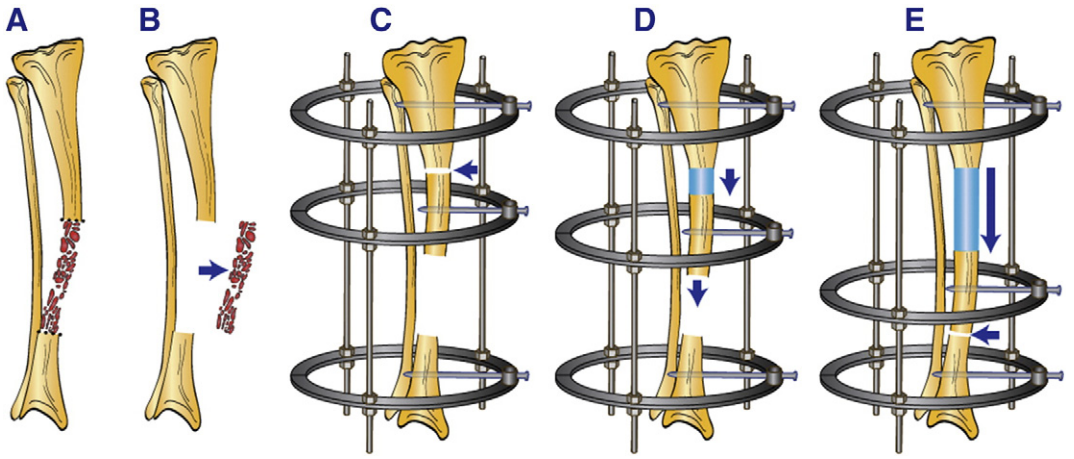

Figure 3. Description of bone transport technique. **(A)** Showing the segmental bone defect. **(B)** Removal of the bone defect. **(C)** Application of the external fixator and performing a proximal tibial osteotomy. **(D)** Start of distraction and transport of the healthy segment to close the bone defect distally. **(E)** End of distraction and completion of bone transport.

sustained release of GFs within desirable time frame.[30] In fact, the role of nanoparticles (NPs) in bone tissue engineering is not limited only to drug delivery but also includes enhancing the mechanical properties of scaffolds (composite scaffolds) and establishing fabricated nanofibrous scaffolds with various effects on the cellular function with the aim of supporting the cell growth and differentiation.[1,31,32]

The expression of growth factors during distraction osteogenesis

In this section we provide a brief overview of the GFs expression in DO [for extensive details the reader can refer to a recent review by (Makhdom and Hamdy 2013)].[10]

Several GFs are known to stimulate cellular growth and function, migration, proliferation and differentiation.[33] Of these, bone morphogenic proteins (BMPs) are considered the most promising osteogenic GF as they are the only ones that play a role in the early differentiation process of undifferentiated mesenchymal cells.[10] In our laboratory, we have extensively studied the spatiotemporal expression of BMPs in animal models of DO.[34-38] BMP receptors (BMPRs) functioned as mediators for BMP signaling. These signals are transmitted by smad proteins. Smad 6 and 7 unlike smads 1, 5 and 8, are inhibitors of smad signaling (Figure 5). Interestingly, the experimental studies showed that smad 6 and 7 (inhibitory smads) are dominant during the consolidation phase of DO while smads 1, 5 and 8 (stimulatory smads) are active during the distraction phase.[35-38] This pattern of smad expression correlated with the expression pattern of BMP-2, 4 and 7 and their receptors. The temporal and spatial expression of BMP 2, 4, and 7 proteins in an animal model of DO showed intense staining for these BMPs in the latency period and it was maintained during the entire distraction phase and once the distraction has stopped, BMP[2,4,7] expression gradually disappeared.[34]

The basic FGF plays an important role in neovascularization during DO and its expression was found to be strongest during the distraction phase. VEGF has also a central role in the bone regenerative process, particularly in angiogenesis, and several studies have demonstrated its role in the regulation of chondrocyte activities, chondroblast/osteoclast activities, and osteoblast activities. The expression of VEGF was found to be highest during the distraction phase of DO. Furthermore, since IGF-I is a potent chemotactic factor for osteoblasts, Schumacher et al have investigated IGF-I expression in an animal model of DO and found that periosteal IGF-I increased after two weeks of distraction and ceased gradually when the distraction stopped.[39]

The PDGF has stimulatory effects as a potent mitogen and chemoattractant for mesenchymal cells, including osteogenic cells. PDGF can regulate the bone regenerative process through other GFs, in which, it increases VEGF expression, enhances IGF-I signaling, and increases expression of various BMP antagonists. Finally, experimental studies showed promising results in terms of enhancing bone regeneration when these GFs were administrated locally in animal models of DO.[10,40-43] These facts have improved our understanding when considering co-administration of GFs to accelerate bone regeneration in DO.

Nanoparticles in growth factors delivery

The value of nanoparticles as growth factor delivery system

The optimal GF delivery system needs to meet specific properties. These include the biocompatibility, biodegradability, non-toxicity, low immunogenicity and the ability to overcome the inherent limitation of the bimolecular therapeutics (e.g. short half life).[44,45] The GF delivery can be achieved through different strategies such as the delivery of the protein itself, or through cells releasing the protein, and genes encoding the protein. Another strategy that leads to the same effect is nanoparticles' use in the delivery of small interfering RNA (siRNA) which increases active GF concentrations by binding the mRNA of GF inhibitors and preventing the inhibitors' expression.[46]

The major conventional classes of delivery materials include natural polymer (e.g. collagen, fibrin, alginate and chitosan), synthetic polymers [e.g. poly (lactic acid), poly (glycolic acid)], inorganic materials (e.g. calcium phosphates, silicate glasses,

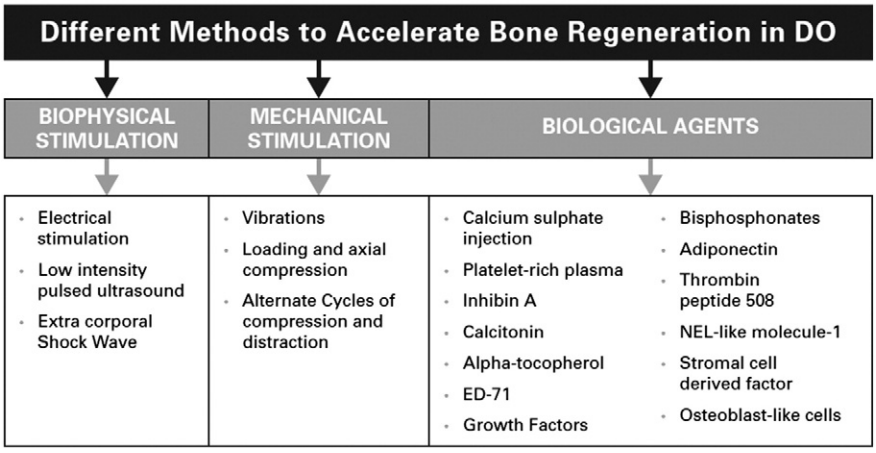

Figure 4. Illustrating different approaches to accelerate bone regeneration in distraction osteogenesis. Modified and adapted from Makhdom and Hamdy 2013.

hydroxyapatite, ceramics and calcium sulfate) and composites which are selected combinations of the aforementioned delivery materials.[47,48] Although these delivery systems have produced more effective delivery of GFs and have reduced the required doses when compared with local application of GFs alone, several limitations and concerns still exist when translating these techniques to the clinic. These include possible transmission of pathogens and immunogenicity (e.g. natural polymers and viral gene), oncogenic risk (e.g. viral gene therapy) brittleness (e.g. inorganic materials) and inflammatory response secondary to the drop of pH (e.g. synthetic polymers).[47,49] NPs can overcome some of these challenges (listed in Table 1).[1,30,45,50-52] Owing to their nano size, NPs have the ability to penetrate deep in tissues without causing damage to the surrounding cells. This property has a major advantage in DO as it will ensure the delivery of drug therapeutics in various parts of the distraction zone and therefore improve the treatment outcome. A final important feature that contributes to the value of nanoparticles is the ease of modulating their polymeric structure that results in excellent control on GFs/gene release kinetics to the target site.[51]

Considerations for using a nanoparticulate system for growth factor delivery

General considerations should be taken into account when delivering GFs with an NP system. The NP fabrication process has to be performed under mild conditions as high temperature or pressure, harsh organic solvent and extreme pH might result in denaturation or deactivation of the integrity of the GFs.[30,53,54] The interaction between phases should be also considered if the incorporation process depends on the affinity of the GFs to the lipophilic phase of the polymer or an emulsion.[54] Furthermore, the particle size has a significant impact on the cellular uptake.[55] It is recognized that 20-200 nm is the most suitable size when delivering therapeutics. This is because larger size particles are rapidly cleared from the circulation due to their uptake by the reticuloendothelial system, while smaller size particles will cross the fenestrated hepatic systems and accumulate in the liver rather than residing for a longer time in the circulation.[56-58] Finally, several factors can determine the release kinetics of the GFs such as the physiological half-life of NPs, stability of NPs in vivo and the loading mode and diffusivity of GFs.[59]

Since the therapeutic drug can have negative effects when delivered through an NP system and released to non-target organs, a method to ensure that NPs can accumulate and stay at the defect site or the skeletal system is necessary. Hydroxyapatite is uniquely present in the extracellular matrix of bone and therefore attaching or adding moieties to NPs that preferentially adsorb to hydroxyapatite is the main strategy used currently to allow bone targeting.[60] Two moieties are used and these are negatively charged oligopeptide sequences and bisphosphonates.[61,62] Sahana et al have investigated NPs of hydroxyapatite loaded with risedronate for targeted bone drug delivery in postmenopausal osteoporotic rat model. The results have shown that significant increase in bone density and decreased bone porosity in the treated rats when compared with controls. Additionally, mechanical bone testing of the treated rats showed significantly greater maximum stress and Young modulus values than controls. The histological analysis showed greater and more organized bone growth.[63] Salerno et al examined the conjugation of alendronate to poly (D,L-lactideco-glycolic) acid NPs and loaded it with doxorubicin (an anticancer drug). This technique allowed the accumulation of the drug at bone and the reduction of the incidence of bone metastases in mice.[64] Other examples of bone targeted delivery include the use of cationic liposomes with six repetitive sequences of Aspartate Serine Serine (poly(Asp Ser Ser)$_6$) attached to their surface. Zhang et al have loaded these liposomes with siRNA for a negative regulation of bone growth. Then they administered these intravenously into rats. The authors have shown that the liposomes accumulate preferentially in bone tissue and cause the least loss of bone in osteoporotic rats.[65] These concepts also should be considered when delivering GFs with an NP system for bone tissue engineering applications including DO.

Types of nanoparticle systems for growth factors delivery

Several forms of NPs systems are available for drug and GFs delivery including biodegradable polymers, lipid based system, inorganic, carbon nanotubes and composites (Table 2).[66-72]

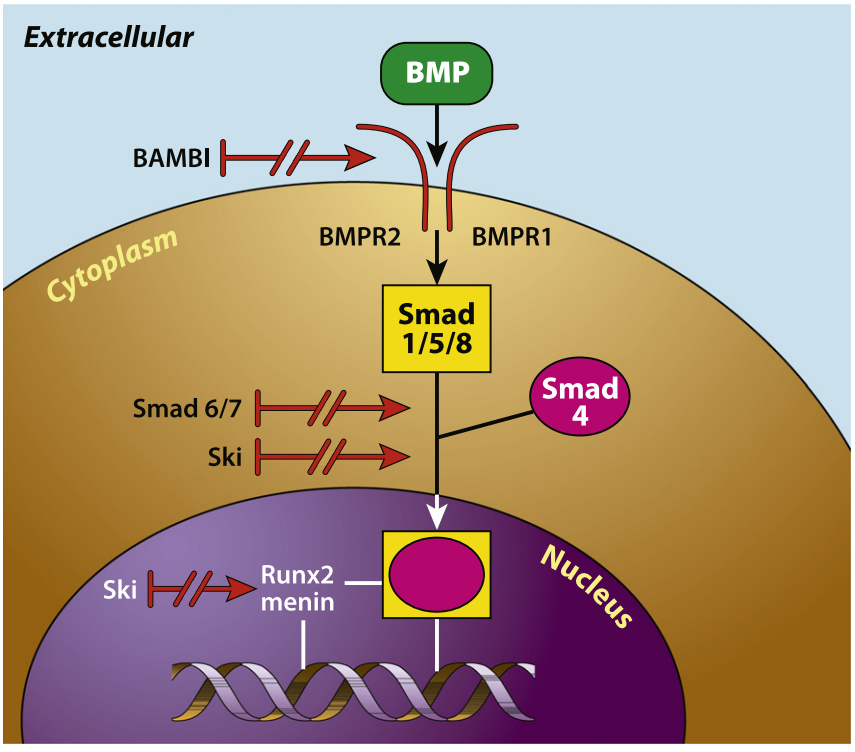

Figure 5. Smad proteins and their role during the BMP signaling pathway.

The polymeric NPs are the most extensively studied in drug delivery and come in several formulas such as nanospheres, nanocapsules, dendrimers and micelles. This is due to the fact that polymeric NPs are highly biodegradable and biocompatible.[56,73-75] In general, the synthetic polymers (e.g. polyglycolide copolymers, polyacrylates and polycaprolactones) have an advantage over natural polymers (e.g. chitosan, gelatin, alginate and collagen) in terms of their ability to achieve more sustained release of drug therapeutics.[56,67] Poly (D,L-lactic-co-glycolic acid)/(PLGA) is a promising synthetic NP polymer that has been studied extensively in specific bone applications. It has a hydrophilic surface with numerous carboxylate end groups.[76] These groups can be modified to achieve prolonged release of drug therapeutics. In addition, PLGA NPs can be conjugated to molecules that have high affinity to calcium phosphate in bone (e.g bisphosphonate and tetracycline) and therefore produce the ability to target more specific bone sites.[77,78] Moreover, PLGA NPs are good candidates for non-viral gene delivery in bone tissue engineering. They have the ability to offer a protection to genes from degradation, increase DNA uptake and subsequent gene transfection and expression.[31] Chitosan is a unique natural polymer that offers several advantages over other copolymers in drug and gene delivery. These include non-toxicity, biodegradability, and make NPs chitosan one of the most interesting materials for the controlled release applications using NPs.[79] In addition, chitosan can be modified chemically and physically to further improve its circulation time, biodistribution, DNA condensation and targeting abilities.[79]

NPs made of inorganic materials also have distinctive features that enable them to extend the novel applications in bone tissue engineering. These features include the chemical similarities to natural bone, and also the electrical, optical, magnetic and mechanical properties.[31] In contrast to polymeric NPs, inorganic NPs have a significant impact on the drug release kinetics and this is related to their longer biodegradation period.[31] NPs made of calcium sulfate have been used to deliver PDGF and have shown superior results in terms of enhancing bone regeneration when compared to PDGF injections alone.[72]

Lipid-base systems include liposomes, lipid nanocapsules (LNC) and solid lipid nanoparticles (SLN). LNC contains a liquid lipid core while SLN contains a core of solid lipids.[80] Liposomes are self-assembled closed vesicles composed of lipid bilayer.[81] These vesicles are formed by bilayers of hydrated phospholipids, which enclose an aqueous core.[82] In fact, these features enable liposomes to be non-toxic and biocompatible. In addition, it allows liposomes to entrap both hydrophilic and hydrophobic drugs as oil-soluble drugs reside within lipid bilayers while water-soluble drugs are entrapped in the aqueous core.[30] To achieve an extended half-life in the circulation, lipid-based systems are often coated with antifouling agent such as PEG.[52]

Carbon nanotubes are carbon cylinders composed of benzene rings. It can shuttle various molecules into the cell such as peptide, proteins and siRNAs via endocytosis.[83,84] Carbon nanotubes exhibit exceptional mechanical, thermal and electrical properties.[85,86] However, since it is insoluble in all solvents, this has generated toxicity concerns. But with the recent advancement in chemical modifications of carbon nanotubes these concerns have been addressed.[87]

NP composites are combinations of different NPs delivery systems. These combinations have been used to optimize the

Table 1
Nanoparticles advantages and properties in drug delivery.

Properties of nanobiomaterials	Biological advantage(s)
Better transport across cell membrane	Decrease the clearance rate from the circulation and therefore enhancing more targeted delivery
Enhancement of mechanical properties of inorganic materials	Can mimic the composition of the natural bone
Larger surface-area to volume ratio	Improved drug bioavailability and loading ability
Nano scale size	(1) Not recognized by the immune systems and therefore provide low immunogenicity. (2) Great dispersion and dissolution power. (3) Penetrate between tissues to reach the target cells without causing tissue damage. (4) Allows internalization and targeting subcellular structures such as nucleus and mitochondria. (5) Greater cellular uptake.
Ability to target certain identified tissues with minimal distribution to normal tissues	Improve the specificity of the drug and open the possibility to address the failure of traditional therapeutics.

benefit from each system. For example, polymeric NPs are biodegradable and biocompatible while inorganic materials have distinctive physical properties that mimic the natural bone. Combining these materials into a single composite NP delivery system will result in synergistic effect on bone regeneration.[73]

The role of nanoparticles in gene delivery

Bone regeneration can be enhanced by the delivery of genes that encode osteogenic GFs through DNA delivery.[46] In addition, using siRNA delivery, silencing of genes that have negative regulatory functions on bone growth such as GF inhibitors can be achieved.[88] Many methods of transfection are available including physical, viral and chemical techniques. Despite the high transfection efficiency of viral methods, there are several associated risks such as activating the body's immune system, activating oncogene expression, genetic recombination of the virus's genome with another virus and limited gene carrying capacity. Transfection with NPs is a chemical method that can avoid many of these problems and allow both ex vivo and in vivo transfection to increase bone regeneration.[89] Owing to their small size, NPs offer the ability to internalize into the cell without alerting the immune system and with no oncogenic risk expression. Additionally, NPs have the ability to protect the DNA by escaping the endosome/lysosome degradation.[89]

Nanoparticles have sizes comparable to molecules naturally uptaken by the cell.[31] Uptake of the nanoparticles occurs by different endocytotic pathways that place the NPs in endosomes to allow their transport from cell membrane to cytoplasm. Early endosomes become progressively more acidic with time, and may finally fuse with lysosomes that degrade all the vesicle's contents. Nanoparticles protect genes from the harsh conditions in endosomes and lysosomes and can be designed to allow endosomes to rupture releasing the nanoparticles into the cytoplasm where they continue their journey and deliver genes to the nucleus. Unprotected genes degrade inside endosomes/lysosomes before they are even released to the cytoplasm making delivery inefficient.[90] Figure 6 shows a summary of the main stages of DNA NP gene delivery system that undergoes release before reaching the nucleus. Delivery of siRNA also benefits from the enhanced cellular uptake and protection effects of nanoparticulate delivery. Unlike DNA, siRNA's final target destination is the cytosol, where it binds to a protein complex (RNA-induced silencing complex, RISC) that helps it binds to the complementary undesired mRNA and degrades it, thereby lowering the translation and expression of the undesired protein (usually an inhibitor of a growth factor needed for bone growth).[91]

In DO, the distraction gap is mainly filled with mesenchymal stem cells, osteogenic cells like osteoblasts and osteocytes, and cells that make new blood capillaries.[92] The transfection of any of these cells with genes that are normally upregulated in DO is sufficient to accelerate ossification. Although the delivery of genes at the distraction gap using nanoparticles has not been attempted, it is likely that the cells at the distraction gap will benefit greatly from such treatment. The delivery of genes for chemotactic factors that increase the recruitment of mesenchymal stem cells can be beneficial at latency and early stage distraction where most recruitment of cells with osteogenic potential occurs. Growth factor genes that increase differentiation of mesenchymal stem cells to osteogenic cells and increase the osteogenic activity of osteoblasts (eg BMP-2 and BMP-7 genes) can accelerate mineralization during the distraction/early consolidation phase. The delivery of genes for angiogenic factors like PDGF or VEGF during any stage of DO is likely to accelerate mineralization by speeding up the growth of capillaries which provide osteoblasts with nourishment that increases their osteogenic activities. Sequential delivery of genes by nanoparticles also has the potential of enhancing DO results. Injection of two types of nanoparticles for example, with one nanoparticle type releasing genes faster than the other due to lower coat thickness or a more permeable shell, may allow the achievement of sequential release, like the release and expression of BMP-2 genes before BMP-7 genes which was shown by several studies to have a synergistic effect on osteogenic mesenchymal stem cell differentiation.[93] In complications of DO when a callus does not form, nanocomposite scaffolds consisting of a 3D matrix that entraps genes, carries nanoparticles the entrap genes, or is seeded with osteogenic cells that were transfected by a nanoparticulate gene delivery system can be implanted to accelerate bone regeneration similarly. Several reports have confirmed the advantages of gene delivery on bone regeneration in general (Table 3).[65,94-98] These are encouraging results for the future use of NPs gene delivery of GFs to enhance bone regeneration in DO. In the next section, we will discuss the applications of nanocomposite in bone tissue engineering.

Table 2
Nanoparticle systems for growth factors delivery.

Nanoparticle system	Structure	Example from a previous study	Biological observation	Author(s) and date
Polymeric micelles	Typically are 10-100 nm sized self assembled polymers and have inner hydrophopic core and hydrophilic outer shell	Heparinized polymeric micelle was used as injectable carrier for bFGF	Long term delivery of bFGF was achieved (over 2 months) in a controlled manner in vitro	Lee et al 2007
Polymeric nanospheres and nanocapsules	Nanospheres: Matrix systems in which the drug dispersed throughout the nanoparticles Nanocapsules: Vesicular systems in which the drug is confined to a cavity and surrounded by a polymeric membrane	PLGA nanoshperes were loaded with IGF. Then, were prepared with either solvent evaporation/ double emulsion or salting out process	The release rate was increased steadily for 24 h and then plateaued for 40 days around 70% when prepared with evaporation method. A little effect was noticed when prepared with salting out method.	Eley and Mathew 2007
Dendrimer	Hyperbranched synthetic polymer with many arms emanating from a central core	EGF* molecules were coupled to a fluorescein-labeled polyamidoamine dendrimer. This conjugate was made to investigate its effect on epidermal growth factor receptor (EGFR).	Dendrimer-EGF conjugates served as EGFR superagonists when compared to EGF alone	Thomas et al 2008
Liposomes	Self-assembling closed colloidal structures composed of lipid bilayers	A comparison of efficacy between the use of liposome-mediated and adenoviral gene transfer was performed for the generation of autologous BMP-2 in a rat bone defect model.	Both groups have shown complete bone healing at 6 weeks when compared to the control groups. However, the liposome-mediated gene transfer was easier in preparation and theoretically less immunogenic.	Park et al 2003
Carbon nanotubes	Well-ordered, hollow structures with excellent mechanical strength. The carbon cylinders composed of benzene ring.	To investigate the highly crystalline multi-walled carbon nanotubes (MWCNTs) on bone healing, and ectopic bone formation when combined with BMP2 and type 1 collagen.	MWCNTs have shown that it is highly compatible with bone tissue, integrated within the bone, induced little inflammation, permit bone repair and accelerated bone formation when combined with BMP2	Usui et al 2008
Inorganic	These materials have a chemical structure that mimic the inorganic materials of natural bone. E.g. ceramic, calcium sulfate, hydroxyapatite (HA) and carbonate apatite	NPs of calcium sulfate was used to deliver PDGF* and to investigate its effect on bone regeneration	The delivery of PDGF by NPs of calcium sulfate have shown superior results in terms of enhancing bone regeneration when compared with PDGF alone	Park et al 2007
Composite	Combination of different NPs delivery systems	Magnetic liposomes with incorporated (rhBMP-2) were prepared to investigate its efficacy on bone regeneration after local injection with implanted magnet in vivo	Local magnetic rhBMP-2 liposomes and magnetic implantation at the injury site was effective for the treatment of bone defects.	Matsuo et al 2003

* EGF: Epidermal growth factor; PDGF: Platelet derived growth factor.

Nanocomposite scaffolds

Nanocomposite scaffolds are scaffolds with at least one component constituent made of nanoscale-sized material. In bone tissue engineering, nanofibers and nanoparticles are common nanoscaled constituents, with collagen fibers and hydroxyapatite crystals as frequently used examples.[99] The nanocomposite scaffolds are often used as two dimensional (2D) scaffolds which are coatings and films that stretch in 2 dimensions and allow tissues to grow on their surfaces only, or three dimensional (3D) scaffolds that consist of matrices that allow cells to grow in three dimensions (on the scaffolds' surface and within it).[100]

Nano-scale features and topographies in 2D nanocomposite scaffolds covering the implants promote bone regeneration by helping in drug delivery, cell attachment and growth, or/and mechanical adherence of the implant's surface to adjacent bone. Coating of implants with nanocomposite 2D scaffolds modifies the surface of the implant so that it has both the required bulk properties such as strength and desired surface properties. Heparin has been conjugated and used as a coating that allows growth factor entrapment and delivery from implants that use it as a coating surface. Other designs use coatings with collagen nanofibers that aid the attachment of cells to the coated implant.[101] Nanocomposite 2D scaffolds are also often used for coatings to allow for very strong adherence through mechanical interlock (Figure 7).[102] The success of any synthetic implant is largely dependent on their ability to achieve osseointegration (the ability to maintain firm adherence with bone at the bone/implant interface). Without this property the synthetic graft remains unconnected to growing bone.[103] The nanometric sized surface texture of 2D nanocomposite scaffold

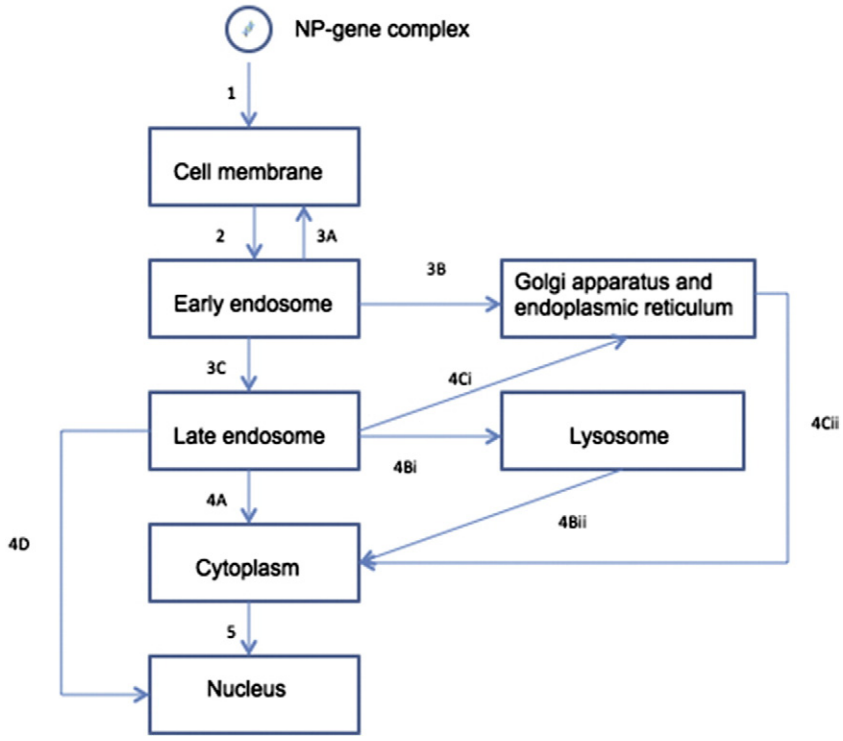

Figure 6. Flowchart showing the journey of a nanoparticle (NP) gene delivery system.[1] Binding of NP to cell membrane. (2) Endocytosis. (3) After the incorporation of NP into early endosome several pathways are possible such as (3A) exocytosis back to extra cellular matrix (3B) uptake by Golgi apparatus and endoplasmic reticulum (3C) NP stays in endosomes as they transform to late endosomes due to the accumulation of H^+ ions by ATP pumps. (4) Escape from late endosome by the following pathways: (4A) agents added to NP help NP to disrupt and break the late endosomal membrane getting released to the cytoplasm, (4B) or disrupt the membrane once late endosomes fuse with lysosomes, (4C) late endosomes may also fuse to Golgi apparatus and travel through endoplasmic reticulum releasing the NP to cytoplasm and bypassing fusion with lysosome, (4D) late endosomes may also be transported using the cytoskeleton to locations near the nucleus allowing theuptake of endosome-NP complex by the nucleus. (5) Uptake of NP/genes released from NP into the nucleus: this occurs by active uptake using nuclear localization signal ligands that can be attached to NPs' surfaces, or during mitosis when nuclear envelop disintegrate allowing entry of gene/NP.

used to achieve adherence by mechanical interlock also allows greater numbers of cells to adhere at the interface.[104] This produces bone that solidifies quicker locking the two interfaces faster and allowing quicker achievement of a stable osseointegrated implant. Additionally, studies have shown that coating with nanocomposite 2D scaffolds enhanced osseointegration more than microcomposite 2D scaffolds. This is related to the fact that microcomposite 2D scaffolds have lower surface roughness when compared to nanocomposite 2D scaffolds.[101,104] Although 2D nanocomposite scaffolds play an important role in implant coating, they receive less attention for tissue engineering when compared to 3D nanocomposite scaffolds. When compared with 2D scaffolds, 3D scaffolds allow more cell proliferation, differentiation and activity of cells that grow in them.[105] A study on human embryonic mesenchymal stem cells showed that their differentiation and osteogenic activity was significantly enhanced when they were allowed to grow in a 3D scaffold rather than a 2D scaffold.[106] Growth on 2D scaffolds forces cells to adhere and spread in a very different way than they do in their natural 3D environments, which affects their shape and ultimately their differentiation. The 2D scaffolds also force growing cells to have fewer cell–cell interactions and different solute (nutrient, gas, growth factor) diffusion/transport patterns. This along with many other restrictions forced by 2D scaffolds changes the cues the cells experience from those in their natural environment and causes less of the expected differentiation.[105] These advantages cause the current research to focus more on the use of 3D scaffolds.

Three dimensional nanocomposite scaffolds have a strong ability to regenerate large volumes of bone owing to their greater resemblances to natural bone components. These natural components include collagen fibers (50-500 nm), compromising 90% of the extracellular protein matrix in natural bone, and hydroxyapatite crystals (few nanometers), compromising the inorganic matrix.[107] Proteins in such matrices adsorb to collagen fibers and link to proteins expressed at the cell's surface allowing firm cell adherence to the collagen fibers. Nanofibers in 3D nanocomposite scaffolds work similarly by allowing protein adsorption which leads to anchorage of cells to scaffolds.[107] The incorporation of NPs in these 3D nanocomposite scaffolds allows better mineralization by providing nanometric surface roughness that enhances protein adsorption and subsequent cell

Table 3
Studies on the application of NPs in gene delivery to enhance bone regeneration.

Method of gene delivery	Delivery system	Genes delivered	Biological response	Author (s) and date
Cells transfected ex vivo then implanted	β tricalcium phosphate ceramic porous scaffolds with mesenchymal stem cells transfected with liposomes-DNA NPs	DNA code for bFGF	Greater and faster bone formation occurs in rabbits with radius bone defects, with greater and more developed blood capillaries	Guo et al 2006
	Collagen sponge with poly(glycolic) acid fibers and acetylated PEI-DNA NPs	DNA code for BMP-2	A significant increase in regenerated bone volume, bone mineral density, BMP-2 expression and Alkaline phosphatase and osteocalcin levels in Wistar rats	Hosseinkhani et al 2007
Cells transfected in vivo	Alginate hydrogel with calcium phosphate-DNA NPs	DNA code for BMP-2	Bony tissue observed at treatment site as early as 2.5 weeks only with scaffold that incorporates NP-DNA	Krebs et al 2008
	Poly(lactic co-glycolic acid) PLGA scaffold with PEI-DNA NPs	DNA code for BMP-4	At least 4.5× increase in bone and mineralized tissue areas and significant increase in BMP-4 expression. Greater volumes of regenerated bone in Lewis rats with cranial bone defects	Huang et al 2005
siRNA delivery	Liposome-siRNA NPs	Chordin siRNA	3× and 2× greater alkaline phosphatase activity and calcium deposition	Kwong et al 2008
	Modified liposome-siRNA NPs	Plekho1 siRNA	Treated Sprague Dawley rats showed the lowest drop in bone volume and bone mineral density	Zhang et al 2012

adherence. NPs incorporated into 3D composite scaffolds can also act as false nanometric nuclei/templates on which hydroxyapatite crystals are aligned allowing accelerated mineralization (Figure 8).[108] After cell adhesion, the pores in 3D nanocomposite scaffolds allow nutrient and waste exchange as well as vascularization and cell migration.[109] The constituents of the scaffolds finally biodegrade allowing bone to replace the gap. Woo et al have shown that bone growth in nanofibrous scaffolds increases cells' attachment by a factor of 1.7 when compared to solid wall scaffolds.[110] The authors then also observed that the osteogenic activities of adhered cells were enhanced in nanofibrous scaffolds.[111] Others have found that the ability of nanofibrous scaffolds to induce differentiation of stem cells into osteogenic cells was higher than solid walled scaffolds.[112,113]

Three dimensional nanocomposite scaffolds have also shown promising success in controlled drug delivery applications. These drugs include GFs or GFs' genes that enhance the osteoinductive potential of cells (ability to induce migration, proliferation, and differentiation of osteogenic cells). Incorporation of GFs into 3D nanocomposite scaffolds can lead to the development of hierarchically organized and multifunctional constructs. These have greater ability to control and guide bone regeneration through the recapitulation of spatial and temporal microenvironments presented by the extracellular matrix.[114-116] The GFs can be physically adsorbed or chemically conjugated to the nanofibers. It can also be physically entrapped in nanofibrous mesh of the 3D nanocomposite scaffold.[117] Interestingly, researchers were able to link peptide sequences that mimic the receptor domain of GFs to nanofibers. This property allows for GF preservation and protects them against denaturation. GF genes with cDNA directly entrapped into the nanofibrous mesh or into nanoparticles in the nanocomposite scaffold can be also used to build up gene-activated matrices.[118] The availability of these different methods of incorporation allows the achievement of a variety of different release kinetics.

The potential roles of nanobiomaterials in distraction osteogenesis

The three key components of bone tissue engineering are composed of biological bioactive agents (e.g. GFs) to offer instructive signals that direct cell growth, mesenchymal or progenitor cells to produce new bone cells and scaffolds to work as transient frame to support bone growth.[31,119] However, these methods have limitations such as brittleness (e.g. scaffolds), short half-life (e.g. GFs) and inability to maintain cell growth (e.g. stem cells). Since the bone microarchitecture is a nanocomposite of hierarchically arranged collagen fibrils, proteoglycans and hydroxyapatite, using nanotechnology in DO would overcome the limitations of these methods as it will maximize their resemblances to natural bone. The potential use of nanobiomaterials in DO is summarized in Figure 9.

Release of single growth factor

Successful entrapment of GFs within core-shell NPs via layer-by-layer (L-b-L) self-assembly technique was recently shown by our group.[120-123] The encapsulation of drug efficiency was enhanced with the increased stability of polyelectrolyte systems. This was achieved through the alternate adsorption of several layers of natural polymers, negatively charge alginate and positively charge chitosan on positively charge nanosized phospholipid vesicles. The L-b-L deposition technique on liposomes produced a spherical, monodisperse and stable composite NP protein delivery system with a cumulative size of 300-400 nm for five bilayer coated liposomes.[120] Loading these composite NPs with BMP-7 was non-toxic with the ability of sustained release of BMP-7 for a prolonged time (45 days) in vitro.[121] This NP system was also biocompatible in vivo[122] and enhanced the bone formation in a rabbit model of DO after a single injection of this composite NP delivery system loaded with

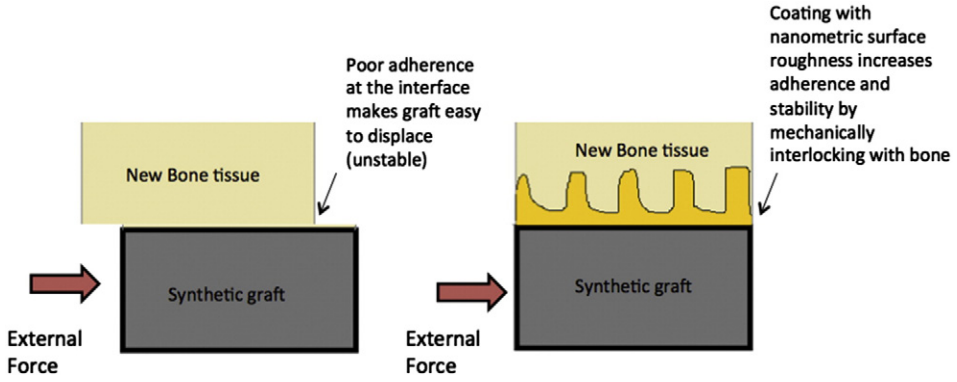

Figure 7. Adherence of the synthetic graft to the surrounding bone is essential for creating a stable graft that does not get displaced by outer stresses/loads. A smooth surface adheres poorly into existing bone **(A)**, however, when the synthetic graft's surface is coated with a 2D nanocomposite scaffold that has nanometric surface roughness, bone fills the crevices and pores of the scaffold mechanically interlocking the two interfaces together and allowing firmer adherence and stability **(B)**.

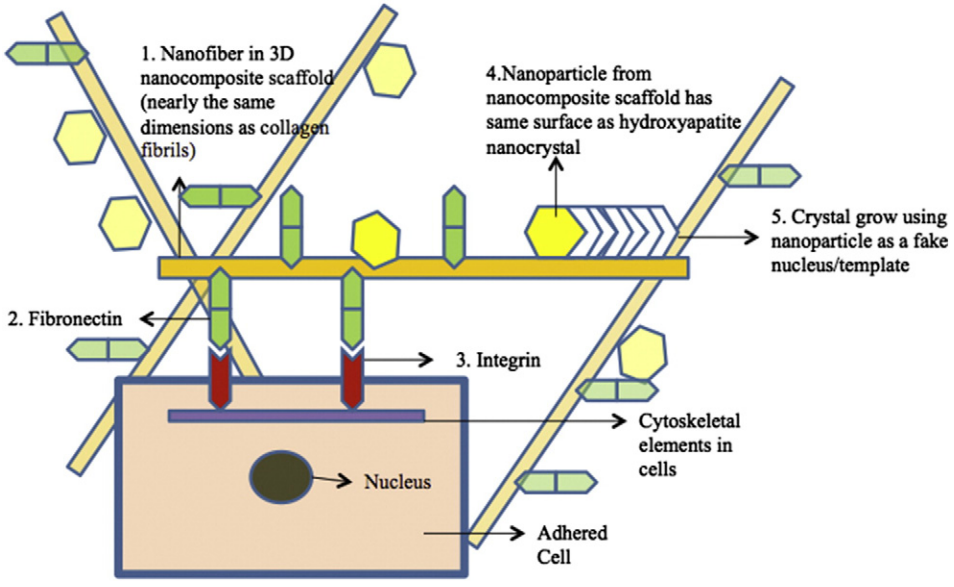

Figure 8. Schematic explaining how 3D nanocomposite scaffolds consisting of nanofibers and nanoparticles trick the body into reacting to it as if it is the extracellular matrix in natural bone tissue (consists of nanometric collagen fibers and hydroxyapatite crystals): 1. The nanofiber is mistaken for a collagen fibril leading to the adsorption of proteins such as fibronectin. 2. Fibronectin adsorbs at one end to the nanofiber and attaches to a passing cell's integrin (a plasma membrane protein used to allow cell adherence). 3. Integrin binds at extracellular end to fibronectin and at the cytoplasmic end to cytoskeletal elements such as actin fibers. This causes the cell to adhere to nanofibers as if they were collagen fibrils. 4. Meanwhile nanoparticles incorporated into the 3D nanocomposite scaffolds are mistaken for nanocrystals that have the same surface dimensions. 5. The nanoparticle's surface is mistaken for the hydroxyapatite crystal surface and growth occurs faster with the nanoparticle acting as a fake nucleus. The end result is bone regeneration due to hydroxyapatite crystal growth and action of the adhered cell that differentiates to an osteogenic cell.

low dose of BMP-7.[123] The dose was dramatically decreased from 75 μg to 1 μg with similar outcomes when delivered via this composite NP.

Other studies have indicated the increasing shift toward using small particulates in GF delivery in DO. Wang et al have investigated the application of nerve growth factor beta (NGFb) delivered by collagen/nano hydroxyapatite/kappa-carrageenan gels to sites of new bone formation in mandibular DO in rabbit model. The authors found that rabbit treated with NGFb in gel had significantly increased consolidation, maximum load to failure and bone volume when compared to control groups.[124] Cho et al have also investigated the effect of local chitosanmicrospheres encapsulated with human growth hormone (group I) injection in mandibular DO in dog model.[125] This was compared with the local injections of saline [control (group II)], hyaluronic acid (group III) and chitosan-microspheres alone (group IV). At six weeks post surgery, the distraction zone of group I was immovable when compared with the other groups. The authors found that the load to failure was highest among group I (52.1%) when compared with group II (16.1%), group III (34.6%) and group IV (41.5%). The

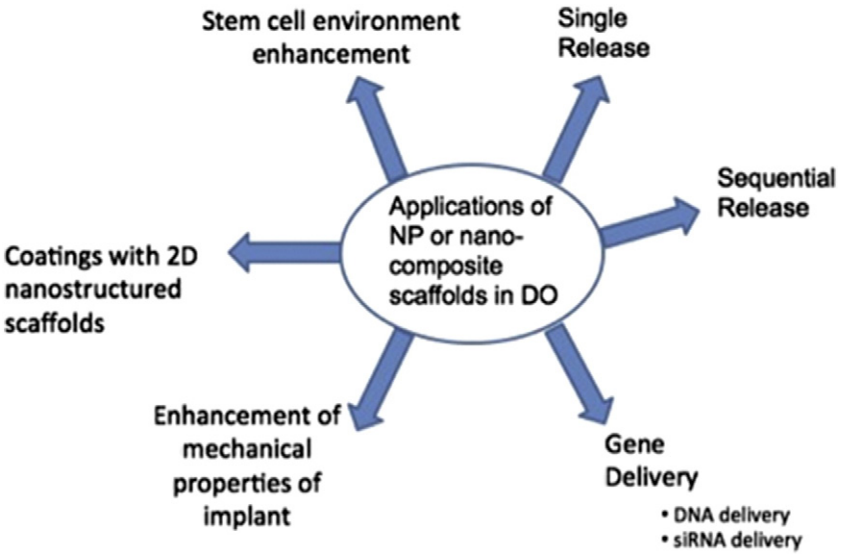

Figure 9. Diagram illustrates the summary of the different potential applications of nanoparticles or nanocomposite scaffolds to accelerate or induce bone regeneration in distraction osteogenesis. Abbreviations: Distraction osteogenesis (DO), nanoparticle (NP), growth factor (GF), small interfering RNA (SiRNA).

load to failure was higher (323%) in group I when compared with the control group ($P < 0.05$). Furthermore, they noted that chitosan microspheres allowed for continuous release of human growth hormone up to 20 days.

These results provided us with the proof that NPs in vivo can be utilized as delivery systems for a prolonged controlled release of GFs in DO at low dose while achieving the desired outcome. Future research may prove that this strategy can be utilized to accelerate bone regeneration in DO in humans (Figure 10, *A* and *D*).

BMP-7 and 2 are the only currently US FDA approved GFs for clinical application. Both BMP-2 and -7 are available commercially for clinical use. These BMPs have been extensively investigated in both animal studies and human trials with many promising results.[33] Consequently, it is reasonable that these BMPs would be the primary targets for nanobiomaterials applications in DO.

Sequential release of multiple growth factors

Reports are available demonstrating that delivery of multiple GFs leads greater biological effects than delivery of a single GF.[126] This is due to the fact that the natural repair in tissues uses many complex cascades with an involvement of multiple GFs in different concentrations and times. Therefore, any treatment aiming to mimic these cascades should not be limited to a delivery of a single GF and should consider delivering multiple GFs at physiological doses and at specific spatiotemporal pattern.[126] In circumstances different than those considered, multiple GFs administration can inhibit bone formation.[127]

Zhu et al have investigated the combined effect of rhBMP-2 and Nell-like molecule-1 (Nell-1) in tibia DO in a rabbit model. The group who were treated with rhBMP-2 and Nell-1 had highest bone volume, peak load and bone mineral density when compared with those who were treated with BMP-2 or Nell-1 alone.[128] Zhang et al have shown that MSC transfected with BMP-2/7 have increased bone formation when compared with control groups in irradiated mandibular DO in rabbit model.[129] Yeh and Lee have also shown that co-transfection of BMP-7 gene and IGF gene stimulated osteoblastic differentiation in vitro when compared to control groups.[130] Jiang et al echoed these results with co-transfection of hBMP-2 gene and VEGF-165 gene.[131] Furthermore, Laflamme et al have shown that epidermal growth factor (EGF) when combined with BMP-2 and/or BMP-7 promotes osteoblast growth in vitro.[132]

These promising results have confirmed the role of co-administration of multiple GFs on acceleration of bone regeneration in DO. However, in order to achieve the desired outcome, sequential and controlled release of these therapeutics is necessary.

Using NP delivery systems or 3D nanocomposite scaffolds, controlled sequential release of multiple GFs with tunable kinetics can be achieved. Since the expression of various GFs has been relatively well studied in DO (as mentioned in section 2.3), our understanding regarding which GFs should we deliver and at what time during DO to accelerate bone regeneration is improved. For example, temporally controlled non-viral gene delivery systems of BMP-2 and 7 in a 3D nanostructured scaffold will be capable to induce a sequential expression of these genes during DO, and aiming to express the gene of BMP-2 during the distraction phase while expressing the gene of BMP-7 during the consolidation phase.

To the best of our knowledge, there have been no previous in vivo studies that have been conducted using NPs or nanostructured scaffolds for sequential and controlled release of multiple GFs in the context of DO. However, some authors have shown promising results toward the use of nano/micro size materials *in vitro* and *in vivo* to promote osteoblast growth and enhance bone repair (Table 4).[133-136] These findings provide researchers with new unexplored area of investigating the nanobiomaterials for sequential and controlled release of multiple GFs in DO.

Environmental enrichment of transplanted stem cells

Long et al have investigated the local effect of bone marrow mesenchymal stem cells (MSCs) which were transfected with

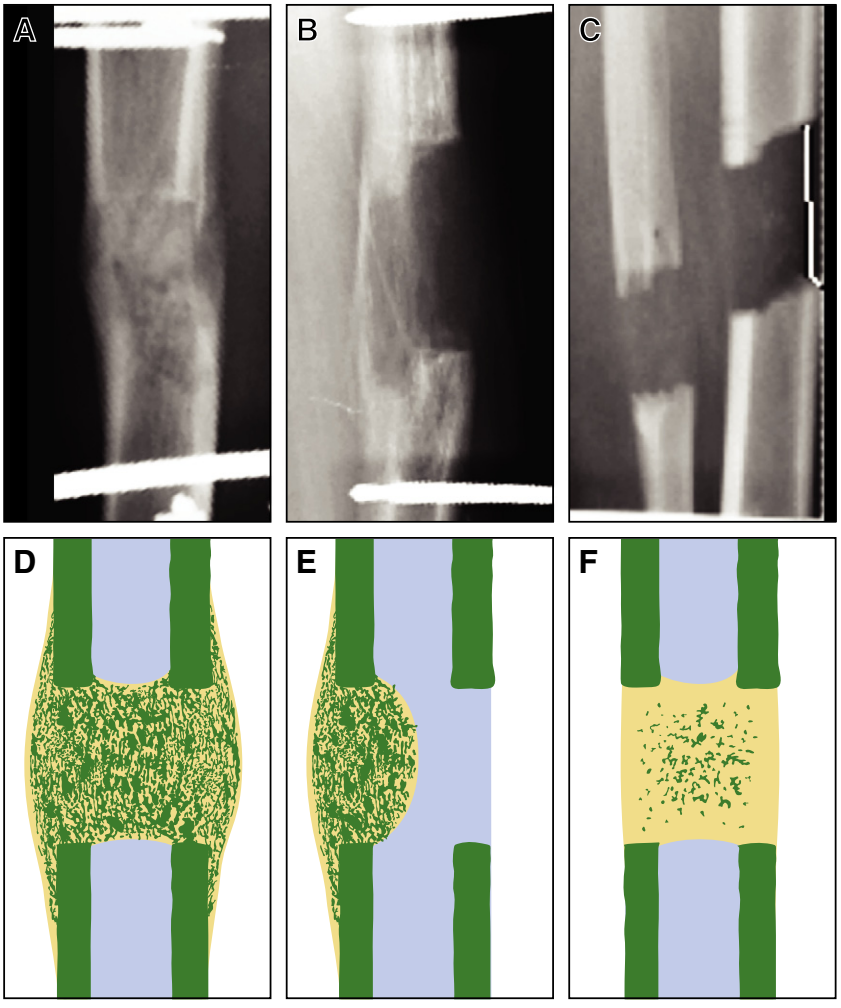

Figure 10. **(A)** and **(D)** Anteroposterior tibial radiographs of a young patient who underwent tibial lengthening. Note that there is good progressive bone formation in the distraction gap. In such circumstances, future advances may prove that the injection of growth factors via nanoparticle delivery systems can accelerate bone regeneration in humans.**(B)** and **(E)** Anteroposterior femur radiograph of a young patient who underwent femur lengthening. Note that there is only bone bridging on the medial cortex of the distraction zone. In such circumstances, future advances may prove that inserting a nanocomposite scaffolds with growth factors may improve the mechanical properties and osteogenic activity of the distracted callus.**(C)** and **(F)** Anteroposterior tibial radiographs of a young patient who underwent tibial lengthening. Note that there is no bone formation in the distraction gap. In such circumstances, stem cells injection in a nanostructured scaffold with or without growth factors can initiate the bone regenerative process.

Adbmp-2 on new bone formation during rapid distraction rate in mandibular DO in rabbit model.[137] They found that this technique has effectively accelerated new bone formation at rapid distraction rate. Aykan et al studied the effect of local injection of MSCs in mandibular DO in sheep model.[138] Bone formation was significantly increased in the treatment group when compared with the control group. These results have shown that MSCs provide a promising future to accelerate bone regeneration in DO. However, the major limitation of stem cells (SCs) is its ability to cultivate and expand in vitro and in vivo. While traditionally the control of SC fate has been attributed to the molecular (e.g. GFs) and genetic factors, nowadays, there is increasing evidence that environmental factors play a paramount role in controlling the fate of these SCs. These factors belong to the nanoscale size materials which could provide surfaces and structures that resemble the natural cellular and extracellular matrix of bone. The nanostructured scaffolds can therefore enhance the SC mobility, adhesion and differentiation during transplantation.[139] Nanofibrous scaffolds are promising environment for cellular in-growth and bone regeneration. This is due to their ability to mimic the extracellular matrix of natural bone, high surface-to-volume ratio and high porosity. They are mainly composed of proteoglycan and collagen.[31] Shin et al have assessed the bone formation from MSC on nanofibrous scaffold in vivo and found that sufficient bone formation was achieved on the surface of the scaffold and type I collagen was expressed.[140] Smith et al have also assessed the effect of 2D and 3D nanofibrous scaffolds on the human embryonic cells. The authors have compared the levels of biological markers that measure the osteoblasts activity (collagen type I, Runx2, and osteocalcin mRNA) between both 2 and 3D cultures and solid walled scaffolds. They found that both 2D and 3D cultures have

Table 4
Previous studies illustrating the role of nano/micro size materials in multiple GFs delivery and bone regeneration.

Growth factors	Nano/microparticle system	Purpose of the study	Biological response	Author (s) and date
BMP-2 and BMP-7	Nanocapsules of poly(lactic acid-co glycolic acid) (PLGA) and poly(3-hydroxybutyrateco-3-hydroxyvalerate) (PHBV). Both were incorporated in chitosan based fiber mesh scaffold.	To develop a nanosized, controlled growth factor delivery system that incorporated in tissue engineering scaffold.	A sequential controlled release of BMP-2 and 7 was achieved. Also a synergistic effect on the osteogenic differentiation of mesenchymal stem cells was observed.	Yilgor et al 2009
BMP-2 and BMP-7	Nanocapsules of (PLGA) and (PHBV) were incorporated in poly(e-caprolactone) 3D scaffolds.	To determine the biological effect on bone regeneration and its relation to the release pattern: each growth factor alone, simultaneous, and sequential.	The greatest alkaline phosphatase activity from MSC was seen with the sequential delivery of BMPs.	Yilgor et al 2010
VEGF and BMP-2	Microspheres of PLGA embedded in a poly(propylene) scaffold surrounded by a gelatin hydrogel loaded with VEGF	To investigate if BMP-2 and VFGF sequential release could enhance BMP-2 induced bone formation	In combination with local sustained BMP-2 release, VEGF significantly enhanced ectopic bone formation compared to BMP-2 alone	Kempen et al 2009
VEGF and PDGF	Composite scaffolds (the core cylinder is a chitosan sponge scaffold intermixed with alginate microspheres and the core cylinder from)	This design intended to allow PDGF delivery followed by VEGF delivery in bone defect model	Greater bone formation was found in scaffold group releasing both PDGF and VEGF when compared with those who had PDGF alone or no GFs	De la Riva et al 2009

expressed higher levels of collagen type I, Runx2, and osteocalcin mRNA when compared with solid walled scaffolds.[141]

In cases with partial/complete absence of bone formation after DO, local administration of MSC loaded in nanofibrous scaffolds theoretically would enhance or achieve bone regeneration (Figure 10, *B* and *E*). To the best of our knowledge, no previous studies have investigated this promising strategy in the context of DO. This is again an excellent opportunity for future experimental research.

Enhancement of mechanical properties of scaffolds

Although the conventional scaffold materials (e.g. inorganic and polymeric materials) are often designed to be biocompatible, biodegradable and osteoconductive, they are limited by their mechanical fragility. Designing scaffolds with nanobiomaterials (as mentioned in Nanocomposite scaffolds section) would enhance the mechanical properties of these scaffolds owing to its resemblance to natural bone. Wei et al have utilized a composite scaffold of nano-hydroxyapatite (NHAP)/poly (L-lactic acid) (PLLA) to establish a composite that mimics the mineral component and microstructure of the natural bone.[142] The authors observed a significant increase in the mechanical properties and the protein adsorption. Xu et al have investigated composite scaffold of nano-fused whiskers/calcium phosphate cement (CPC) to establish a strong bioactive composite that can overcome the brittleness of CPC.[143] They found that the flexural strength, elastic modulus and hardness of the bioactive whisker-CPC composite almost matched those of cortical bone. Furthermore, Hong et al have shown that uniform NHAP/PLLA composites have better mechanical properties when compared with conventional PLLA/HAP composites.[144] They also found that NHAP/PLLA composites have improved cell adhesion and biocompatibility.

In fact, these promising nanocomposite scaffolds can be utilized in DO to provide a mechanical support of the growing callus especially in cases with suboptimal callus formation (Figure 10, *C* and *F*). However, further experimental research should be conducted to explore how and when these nanocomposite-based scaffolds should be inserted.

When suboptimal callus formation in DO is encountered, nanocomposite scaffolds might be useful approach to provide mechanical and biological support in the distraction zone.

Summary and future directions

DO technique is used worldwide to treat many orthopedic and craniofacial complex conditions. However, one major limitation is the long time the fixator is left in place until the bone is completely consolidated. Application of exogenous biological agents including osteogenic GFs is one approach to accelerate bone regeneration during DO. Despite the promising results from the animal data, its use is limited in the clinic. This is secondary to the short-half life, rapid clearance and safety concerns. Therefore, developing an effective delivery system is required. The optimal delivery systems have to be biocompatible, biodegradable, non-toxic, non immunogenic, able to preserve the biological activity of the biomolecules, and overcome the inherit limitations of drug therapeutics. Nanobiomaterials have attractive and powerful properties that enable them to achieve more effective sequential and controlled release of dug therapeutics over other modalities. With the increased understanding of the spatiotemporal expressions of various GFs, new opportunities for future experimental DO research are opened to find the optimal NP delivery system (local, practical and cost effective) or nanostructured scaffold to achieve single or multiple controlled and sequential release of these GFs during DO.

Nanobiomaterials can be used to strengthen the mechanical properties of scaffolds and to control the fate of the transplanted SCs by providing an environment that resembles the natural cellular and extracellular matrix of bone.

Future experimental research should be conducted to answer the following questions: when to inject the nanobiomaterials? And which formula should be used during DO? For example, in cases with progressive bone formation during the distraction phase, probably it is sufficient to inject a single/multiple GFs via an NP delivery system or nanostructured scaffolds to accelerate bone regeneration. While in cases with complete absence of bone formation probably injecting SCs in a nanostructured scaffold with or without GFs is necessary to initiate the bone regenerative process and support bone growth. Furthermore, since the partial knowledge of the internalization pathway and of ligand–target interaction is often the reason for the failure of the delivery system, nanotechnology can be used to identify the full picture of the intracellular interactions of the drug therapeutics and the natural cascades during DO. Finally, future advances in tissue engineering at the nanoscale size promise a new bright avenue for DO.

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