

## A Brief Review of Viral Delivery Systems for Bone Tissue Engineering

Alan Nguyen, Dalton Pham, Vi Nguyen, Jia Shen, Michelle A Scott, Benjamin Levi, Aaron W James

Alan Nguyen, Dalton Pham, Vi Nguyen, Aaron W James, Department of Pathology & Laboratory Medicine, David Geffen School of Medicine, University of California, Los Angeles, the United States

Alan Nguyen, Jia Shen, School of Dentistry, University of California, Los Angeles, the United States

Michelle A Scott, Children's Hospital, Los Angeles, the United States

Benjamin Levi, Aaron W James, Division of Plastic Surgery, Department of Surgery, University of Michigan, the United States

Correspondence to: Aaron W. James, MD, Department of Pathology & Laboratory Medicine, David Geffen School of Medicine, University of California, 10833 Le Conte Ave, CHS A3-251, Los Angeles, CA 90077, the United States

Email: [awjames@mednet.ucla.edu](mailto:awjames@mednet.ucla.edu)

Telephone: +1-415-860 2815

Received: September 30, 2014      Revised: October 24, 2014

Accepted: October 30, 2014

Published online: December 23, 2014

tissue engineering is not necessarily the osteoblast or osteocyte. Other tissue-specific cell targets including intramarrow adipocytes, bone associated endothelial cells, or even adjacent skeletal muscle cells can have a secondary effect on osteogenesis and have been increasingly investigated. In this review, we will focus on the fundamental roles of vectors and gene delivery products, as well as discuss frequently targeted, alternative tissue types in the context of bone tissue engineering.

© 2014 ACT. All rights reserved.

**Key words:** Bone tissue engineering; Gene therapy; Vectors; RNAi; Minicircle; MiRNA

Nguyen A, Pham D, Nguyen V, Shen J, Scott MA, Levi B, James AW. A Brief Review of Viral Delivery Systems for Bone Tissue Engineering. *International Journal of Orthopaedics* 2014; 1(4): 130-139 Available from: URL: <http://www.ghrnet.org/index.php/ijo/article/view/974>

### ABSTRACT

To date, bone tissue engineering remains one of the most challenging fields for both researchers and clinicians. The traditional standard of using autograft or allograft bone introduces a number of complications, including pain, infection, and donor-site morbidity among others, prompting researchers to find alternative solutions. However, with the development of techniques in the field of gene therapy, there is now greatly improved clinical potential for bone tissue regeneration in terms of providing treatment that is both safe and efficacious. While viral delivery is a well-studied platform for gene transfer, non-integrating also represents an attractive alternative method as it addresses concerns of safety. Products for viral based delivery vary widely, from micro (mi)RNAs to bone morphogenetic proteins (BMPs). Finally and interestingly, the target cell for bone

### INTRODUCTION

Autograft bone has long been regarded as the gold standard for bone tissue regeneration, and free bone tissue transfer allows the placement of vascularized bone into a unique region devoid of osseous support. However, a number of complications exist, including flap failure, pain, infection, blood loss, and donor-site morbidity<sup>[1]</sup>. Additionally, allografts suffer from even greater resorption and infectious complications due to the use of decellularized material and a lack of native blood supply. Both autografts and allografts could benefit from gene transfer techniques, which provide an effective local delivery of signaling factors<sup>[2]</sup>. In gene therapy, small sequences of DNA or RNA are delivered to cells or tissues to correct for a genetic defect<sup>[3]</sup>. The goal is to stimulate local tissues mitigating the need for the use of any osseous graft. In fact, numerous diverse methods have been

employed in preclinical studies to modulate expression of growth factors, transcription factors, and other signaling elements to enhance osteogenesis and/or angiogenesis in a bone defect site<sup>[4]</sup>. Only until recently has stem-cell based tissue engineering been combined with gene therapy in a hybrid approach; in this time, studies have shown promising regenerative potential<sup>[5]</sup>. This review will highlight these emerging techniques that aim to improve upon the state of bone tissue engineering while simultaneously circumventing the drawbacks of autograft bone.

Currently, several delivery systems exist, most of which involve plasmid integration. The main objective is to either replace or silence genes to restore, change, or augment gene function. As well, there exist non-integrating technologies that do not carry with them a risk of insertional mutagenesis. In addition to vector type, there are diverse products of delivery, including but not limited to micro (mi) RNAs, transcription factors, and cytokines to enhance osteogenic differentiation and bone formation.

The final key aspect which will be discussed in this review is the direct targeting of bone tissue by gene therapy, as opposed to targeting adjacent and supporting tissues. In the context of genetic manipulation, investigators have explored differentiation of several supporting tissues, including adipocytes, endothelial cells, and skeletal muscle cells. As will be discussed, a unique balance exists between adipogenesis and osteogenesis in which manipulation of one pathway has an influence on the other. Endothelial cells also contribute to the vasculature of bone tissue, a factor that is overlooked in many conventional methods of bone regeneration. Recent works even suggest that endothelial cells themselves can become mesenchymal cells which then can undergo endochondral ossification (endothelial to mesenchymal transition)<sup>[6]</sup>. Lastly, the influence of skeletal muscle on bone healing and bone maintenance is an area of active investigation. Improved understanding of genetic manipulation, via basic components of vector types, delivery products, and tissue-specific targets, may lead to safer and more efficacious models for bone tissue engineering and regeneration.

## VIRAL DELIVERY SYSTEMS

Gene therapy is the process of introducing foreign genetic material into host cells in order to treat or prevent disease. Various viral gene delivery systems have been developed in order to solidify this technique. Viral gene therapy capitalizes on the long evolutionary periods that has enabled their genetic material to integrate into host cells, thus making viral-vectors a popular choice for gene therapy studies<sup>[7]</sup>. With such variety to choose from, each viral vector type comes with their respective advantages and disadvantages. Each of these methods, including retroviral and adenoviral vectors, and silencing RNA technology are discussed sequentially below<sup>[8]</sup>.

### Retroviral Vectors

Retroviral vectors play a central role in gene therapy due to their ability to lead to permanent gene transfer. Specifically, the most important advantage of these vectors is the capability of transforming single-stranded RNA genome into a double-stranded DNA molecule that can subsequently transduce into the host cell's genome<sup>[9]</sup>. Retroviral vectors are able to deliver 8 kb of DNA sequence. Furthermore, they have the ability to permeate nuclear pores of mitotic cells, enhancing their efficacy in *in situ* treatment<sup>[9]</sup>. However, the ability of retroviruses to integrate into the target cell's chromosome also comes with potential risk of insertional mutagenesis and activation of oncogenic programming<sup>[10]</sup>. Oncogenic

retroviruses have been shown to induce malignancy in patients after a latency period; for example, both human T-cell leukemia virus 1 (HTLV-1) and bovine leukemia virus (BLV) have been shown to cause adult T-cell leukemia<sup>[11]</sup>. In 2000, Cavazzana-Calvo *et al* successfully used retroviral vectors to treat patients with severe combined immunodeficiency (SCID)-X1, an X-linked disease characterized by inhibited T and natural killer (NK) lymphocyte differentiation due to a defective cytokine receptor gene. While treatment was successful<sup>[12]</sup>, three of the treated patients subsequently developed T-cell leukemia as a result of retroviral vector activation of the LMO2 oncogene<sup>[13]</sup>. In another study, however, Bosticardo *et al* demonstrated that the use of retroviral vectors had a safe risk-benefit profile with respects to developing leukemia in the treatment of Wiskott-Aldrich Syndrome (WAS), an X-linked immunodeficiency characterized by thrombocytopenia and susceptibility to tumor development. Specifically, researchers utilized retroviral gene therapy to produce and administer WAS gene-corrected autologous hematopoietic stem cell (HSC), leading to clinical improvement and corrected functional defects<sup>[14]</sup>. In both of these early gene therapy studies targeting both SCID and WAS, patients were administered retroviral vectors with long terminal repeats without chemotherapy, and had resulted in the development of T-lymphocyte leukaemia<sup>[15]</sup>. Current trials now utilize new generation, self-inactivating gamma-retroviral vectors, which have so far been successful without any reported serious adverse effects<sup>[15]</sup>. Taken together, there are many potential applications for retroviruses in the field of bone tissue engineering; however, retroviral vectors are only one of several methods for genetic manipulation in this field.

### Lentiviruses

Lentiviruses are a subset of retroviruses that possess the unique ability to undergo transduction with nondividing cells, in addition to dividing cells<sup>[16]</sup>. Like retroviral vectors, they can deliver 8 kb of sequence. A critical advantage, however, is the maintenance of stable, long-term transgene expression<sup>[17]</sup>. Lentiviral vectors have been used in numerous experiments utilizing animal models that aim to treat various disorders. For example, through lentiviral gene transfer, Jaako *et al* demonstrated that enforced expression of ribosomal protein S19 cured anemia and lethal bone marrow failure in murine recipients transplanted with ribosomal protein S19-deficient cells<sup>[18]</sup>. Furthermore, these gene-corrected S19-deficient cells did not exhibit any signs of vector-mediated toxicity. Other investigators have further shown the diversity of applications that lentiviral vectors have in terms of treatment of diseases. Betchen *et al* investigated the efficacy of using lentiviral vectors to replace lost gamma-aminobutyric acid in defective neurons to advance the treatment of Parkinson's disease<sup>[19]</sup>. Meanwhile, Federici *et al* demonstrated that HIV-1 lentiviral vectors pseudotyped with the Rabies PV glycoprotein could be used to target the central nervous system to treat motor neuron diseases<sup>[20]</sup>. With the current development of gene replacement strategies, especially lentiviral vectors, clinical treatment of diseases that were once thought to be difficult, such as of the central nervous system, are now not only possible, but considerably safer and more effective<sup>[21]</sup>. Thus, while similar to other retroviral vectors, lentiviral vectors present an alternative technique for gene manipulation with characteristic advantages that can be utilized in bone tissue engineering.

### Adenoviruses

Adenoviruses also present a unique set of advantages, due to their relatively low host specificity. For example, adenoviruses types 2 and 5 can be used to target both dividing and nondividing

cells<sup>[22]</sup>. Moreover, adenoviruses differ from retroviruses in that they are able to deliver longer DNA sequences up to 38 kb into the nucleus; however, they are unable to permanently integrate into the chromosome of the host cell<sup>[23]</sup>. Consequently, adenoviral gene therapy is usually associated with low levels of pathogenicity since the viral DNA does not remain transduced in the host genome; most patients however do not experience any adverse immunological response<sup>[23]</sup>. In a study by Schiedner *et al.* that found *in vivo* transcriptional regulation to be tissue-specific, human alpha1-antitrypsin (alpha1AT) locus was used with an adenoviral vector lacking all viral coding sequences, which decreases the host immune response to the viral protein<sup>[24]</sup>. In particular, mice receiving this adenoviral treatment intravenously showed stable gene expression, as well as decreased acute and chronic toxicity<sup>[24]</sup>. Use of adenoviral vectors is also showing promise in anti-tumor treatments. In one study, Hara *et al* utilized an adenoviral vector to increase Dickkopf-3 (DKK3) protein to attenuate the expression of Wnt3a, Wnt52, and LRP6, which greatly affected downstream Wnt cascades. Thus, anti-tumor effects were achieved through this suppression of the Wnt pathway in glioblastoma xenograft models<sup>[25]</sup>. Another adenoviral vector cancer treatment application targets the suicide gene; enzymes that metabolize prodrugs to chemotherapeutic agents are delivered into the tumor cells, resulting in apoptosis in neighboring non-transduced cells<sup>[26]</sup>. For example, adenoviral delivery of the herpes simplex virus thymidine kinase (*HSV-tk*) gene, which activates the prodrug ganciclovir, has been experimentally used to treat brain tumors<sup>[27]</sup>. While herpes virus has not otherwise been studied in the context of repairing bone, studies have extensively used adenoviral vectors for bone formation, as discussed below<sup>[28-30]</sup>. Overall, adenoviruses are advantageous given their ability to deliver longer sequences to both dividing and nondividing cells without insertional mutagenesis; however, induced host immune response remains a concern for clinical translation.

### RNA interference

RNA interference (RNAi) has emerged as a crucial pathway currently used to silence harmful or defective genes through the delivery of short interfering RNA (siRNA). This mechanism begins when double-stranded siRNA is processed into short 21-25 base pair units by the Dicer enzyme, and then loaded onto an RNA-induced silencing complex (RISC). After one of the strands is cleaved, the remaining strand binds to a complementary strand of mRNA, which is subsequently cleaved and degraded by nucleases, thus ending translation<sup>[31]</sup>. One of the major setbacks of siRNA is a relatively short duration of therapeutic effect in contrast to DNA gene therapy, as RNA is more susceptible to degradation relative to DNA. siRNAs that are transfected into cells achieve transient gene knockdown for only three to seven days in rapidly dividing cells, and approximately three weeks for nondividing cells<sup>[32]</sup>. Most notably, Hoelters *et al* was the first to successfully demonstrate effective non-viral gene transfer of DNA and siRNA into human (h)MSC. Specifically, gene transfer and silencing was achieved through non-viral DNA transfection of enhanced green fluorescent protein (EGFP) and EGFP-targeted siRNAs<sup>[33]</sup>. Furthermore, these hMSC maintained the ability to proliferate and differentiate into different mesoderm lineages, thereby verifying the use of RNAi in conjunction with viable stem cell platforms for gene transfer (Please see<sup>[34]</sup> for an in-depth review of RNAi application in stem cells). Applied to bone tissue engineering, siRNA has been successfully demonstrated in an osteoporotic model, through targeting of semaphoring-4d (Sema4d), to aid in mitigating bone loss<sup>[35]</sup>. Taken together, RNAi represents a viable platform for

gene therapy applications in bone tissue engineering.

## NOVEL, NON-INTEGRATING METHODS

### Minicircles

While retroviral and lentiviral techniques have been shown to be efficacious methods for genetic modification, there is a primary concern for insertional mutagenesis<sup>[36,37]</sup>. A popular alternative has been the minicircle, which is FDA-approved and has thus far shown significant translational potential for clinical use<sup>[38]</sup>. Minicircles are non-integrating episomal DNA vectors, characterized by a circular expression cassette lacking bacterial plasmid DNA<sup>[38]</sup>. Additionally, the minicircle has been further improved upon through incorporation of circular integrating plasmid, such as an additional recombinase. Its ability to simultaneously deliver short hairpin ribonucleic acid (shRNA) and a GFP label makes it a promising therapeutic tool for future tissue engineering applications. In the field of bone tissue engineering, minicircles have been applied to modulate bone morphogenetic protein (BMP) signaling. In one such study, delivery of Noggin shRNA via minicircle construct was shown to enhance osteogenic differentiation and gene expression, at levels comparable to a lentiviral approach<sup>[39]</sup>. When minicircle treatment was applied *in vitro* human adipose-derived stem cells (ASC), followed by *in vivo* application in murine calvarial defects, investigators observed accelerated healing in contrast to that of minicircle GFP control<sup>[40]</sup>. Minicircles, however, being non-integrating will only remain for a limited amount of time. If the gene of interest only needs to be expressed during early time points, this strategy can be powerful. If, however, continued gene expression is necessary, this technology loses significant potential. In summary, as a non-integrating method with higher transfection efficacy over plasmids and showed improved safety over retroviruses and lentiviruses, minicircles are proving to be an attractive alternative for application in bone tissue engineering.

### Other Non-Integrating Methods

In addition to the minicircle plasmid approach, there exist derivatives among other non-integrating techniques. One approach that has been shown to be successful in bone regeneration is sonoporation, which creates pores in the cell membrane via ultrasound and facilitates uptake of genes both *in vitro* and *in vivo*<sup>[41]</sup>. Using this technique in a rat model, Nishida *et al* demonstrated transgene expression *in vivo* of up to 24 weeks was possible in intervertebral disc cells<sup>[42]</sup>. A similar method is nucleofection, which is a nonviral gene therapy technique utilizing electroporation to allow for uptake of genetic material. Nucleofection has shown a 90% transfection efficiency of adult human mesenchymal stem cells as well as a significantly increased transfection efficiency compared to other techniques, such as electroporation and liposome-mediated gene transfer<sup>[43,44]</sup>. Thus far, it has been implemented in bone regeneration<sup>[45]</sup>, and has even been examined across several animal models<sup>[46]</sup>. For example, electroporation-mediated transfer of *Runx2* and/or *Osterix* genes was found to enhance both *in vitro* and *in vivo* osteogenesis of ASC, as applied in an ectopic murine model of bone formation<sup>[47]</sup>. Likewise, Lee *et al* also observed that cotransfection of the *BMP-2* gene with *Runx2* gene via electroporation increased *in vivo* ASC mediated ectopic bone formation<sup>[48]</sup>. Furthermore, liposome-mediated gene transfer exhibited successful bone regeneration in critical-sized defects in various model systems<sup>[49,50]</sup>. For example, Lutz *et al* was able to demonstrate osseous integration and bone regeneration in a porcine model with calvarial defects after *in vivo* liposomal vector gene delivery of *BMP-2*. Compared to the pigs that

only received a bone graft, pigs in the liposomal *BMP-2*/bone graft group demonstrated significantly higher mineralization rates after 28 days<sup>[50]</sup>. Usage of non-viral vectors is limited by their non-selective cell targeting, low transfection efficiency, and thus, low transgene expression; however, progress in improving efficiency and efficacy is being made. Overall, non-integrating techniques may have improved the safety of gene therapy, but progress for further safety, efficiency, and efficacy must be made before making its way towards clinical applications for bone regeneration.

## DISCUSSION DELIVERY PRODUCTS FOR GENE THERAPY

### miRNA Delivery

In context of genetic manipulation in bone tissue, one of the more promising methods is the delivery of microRNA (miRNA) for bone repair. miRNAs are conserved small non-coding RNAs, approximately 22 nucleotides in length, and found in almost all biological processes<sup>[51,52]</sup>. Only recently have miRNA been shown to be involved in the multilineage differentiation of mesenchymal stem cells (MSC)<sup>[53]</sup>. In osteogenic differentiation of hMSC, 19 miRNAs have been reported to be upregulated; interestingly, 11 of these were also seen upregulated in adipogenic differentiation. Recent microarray analyses have identified a select cohort of miRNAs (miR-204, -138, -30, and -23a) that exert effects on osteogenic differentiation<sup>[53-55]</sup>. Specifically, miRNA-138 has been shown to regulate osteogenic differentiation of hMSC, seen both *in vitro* and *in vivo*, primarily through inhibition of osteoblastogenesis resulting in reduced ectopic bone formation<sup>[53]</sup>. In fact, both osteogenic and adipogenic differentiation of hMSC can be inhibited through use of lentiviruses expressing shRNA to downregulate Dicer and Drosha enzymes, both of which are responsible for early transcription of miRNA<sup>[56]</sup>. However, there are currently very few studies that have examined the effect of miRNA-modified adult stem cells in repairing bone defects.

Studies involving miRs in the context of bone tissue engineering have revealed that a select subset influence bone formation, ossification, and maintenance<sup>[57,58]</sup>, while another cohort specifically plays a role in the osteogenic differentiation of ASC<sup>[59,60]</sup>. For example, miR-2861 overexpression has been shown to promote osteoblast differentiation *in vitro*, while *in vivo* silencing of miR-2861 results in an osteopenic mouse phenotype<sup>[57]</sup>. Applied to humans, miR-2681 is highly conserved, such that a homozygous mutation in the precursor for miR2681 was shown to be associated with primary osteoporosis in two adolescent patients<sup>[57]</sup>. Moreover, miR-29a has been found to be essential for human osteoblast differentiation and acts as a positive regulator of canonical Wnt signaling<sup>[61]</sup>. Other miRs, including miR-196a and -31, have specifically been shown to play a role in regulating osteogenic differentiation of human ASC. When lentiviral expression of miR-196a was increased in ASCs, a significant increase in osteogenic differentiation followed *in vitro*<sup>[59]</sup>. Applied *in vivo*, silencing of miR-31 in ASC was required to increase bone volume and bone mineral density in repair of critical-sized defects<sup>[62]</sup>. Finally, miRNA are also believed to have an essential role in inhibiting MSC osteogenesis; many are either underexpressed (miR-31, -106a, -148a, and -424) or overexpressed (miR-130b, -30c, and -15b) in osteo-differentiated MSCs<sup>[63]</sup>. Predicted targets of these miRNA are diverse, and include CD44, integrin B1, PDGF, among many others<sup>[64-69]</sup>. In summary, the importance and use of miRNA to exert effects on bone formation is a relatively nascent field, showing great promise towards applications in bone tissue regeneration.

### Targeting BMP (Bone Morphogenetic Protein) Signaling

Genetic manipulation for bone tissue engineering is also possible through the regulation of various cytokines, including insulin-like growth factor, platelet derived growth factor, fibroblast growth factor, transforming growth-factor, vascular endothelial growth factor, and bone morphogenetic proteins (BMP)<sup>[3]</sup>. Of these, the most well studied cytokine is BMP. Recombinant BMPs have been shown to induce robust ectopic bone formation and healing of bone defects across different animal models<sup>[70-73]</sup>. As well, they have also been found to induce a variety of other effects such as inducing osteoprogenitor cell migration, proliferation, and differentiation, while simultaneously inhibiting the differentiation of myogenic and adipogenic cells<sup>[3]</sup>. As expected, however, a higher dose of recombinant protein is required than the endogenous levels expressed during bone repair if the objective is to accelerate healing. This is likely explicated by suboptimal delivery vehicles and/or rapid *in vivo* protein degradation. For this reason, gene therapy is an attractive alternative strategy for the sustained delivery of BMPs to a compromised bone tissue site.

Thus far, gene therapy strategies primarily focus on the delivery of *Bmp-2*, *Bmp-4*, and *Bmp-7*, and have proven successful when applied to enhance bone formation in pre-clinical animal models. In particular, injection of adenoviral vectors containing *BMP-2* has been shown to enhance bone formation in critical-sized rat mandibular defects<sup>[28]</sup>, as well as rabbit femoral segmental defects<sup>[74]</sup>. In a murine calvarial defect model, knockdown of the well-established BMP inhibitor *Noggin* in human ASCs, via lentiviral and nonintegrating minicircle shRNA methods, resulted in upregulated BMP signaling and osteogenic differentiation<sup>[39]</sup>. It is worth noting, however, that the majority of these studies utilize the platform of bone marrow stromal cells (BMSCs), which have been genetically engineered to overexpress *BMP-2*. For example, constructs created using *ex vivo* adenoviral *BMP-2* mediated gene transfer to BMSCs led to solid bone formation after a time period of three months in a swine skull defect model<sup>[75]</sup>. Similar findings, also using BMSCs adenovirally transduced with *BMP-2 ex vivo*, were observed in rabbit, mice, and dog maxillary sinus augmentation models, in which increased bone formation was observed over the control group<sup>[76,77]</sup>. In addition to transduction via adenoviral vector, liposomal-mediated delivery of *BMP-2* into BMSCs has been shown to be just as efficacious in healing critical size bone defects, but with the advantages of ease of preparation and less immunological concerns<sup>[49,78]</sup>. Finally, several studies have suggested that muscle-derived osteoprogenitors may play a role in influencing differentiation, as seen by induced ectopic bone formation from intramuscular injection and increased endogenous expression of *BMP-2* around the defect site<sup>[74,79]</sup>. Although clinically undesirable, ectopic bone formation does underscore the robust osteoinductive potential of the *BMP-2* adenoviral delivery combination. From a clinical standpoint, however, one must keep in mind that *BMP-2* cytokine delivery can also stimulate osteoclastogenesis as well as destructive ectopic bone. Recent studies investigating *BMP* application in spinal fusion have shown potential associated complications, including local bone resorption, pseudoarthrosis, osteolysis, and nerve injury<sup>[80]</sup>; early studies in *BMP-2* delivery to spine surgery patients has led to notable side effects including spinal cord compression. In summary, injection of adenoviral containing *BMP* has shown consistent results across several animal models, and even tissue types, showing great promise for clinical translation for gene manipulation.

## TARGETING BONE CELLS VERSUS SUPPORTING CELLS IN BONE TISSUE ENGINEERING

While bone tissue itself seems to be the most direct target for bone tissue engineering, it is possible to stimulate bone regeneration through a number of other supporting tissues. Investigators have explored the indirect osteogenic role of various signaling pathways involved in alternative, supporting and/or adjacent tissue ranging from adipocytes, endothelial cells, and skeletal muscle cells.

### Targeting Adipocytes

As an alternative to targeting bone tissue directly, adipose tissue has been proven to be promising in terms of stimulating new bone formation *in vivo*. It is first important to note that skeletal aging can be explained by a shift in balance between osteoblasts and osteoclasts, but interestingly, increased marrow adiposity also plays a significant role<sup>[81]</sup>. This suggests a relationship between osteogenic and adipogenic differentiation. In fact, investigators currently theorize that an inverse relationship exists between the two, which has thus far been observed in MSCs<sup>[76]</sup>. That is, a predilection towards an osteoblastic phenotype occurs at the expense of adipogenic differentiation. Of course, this balance is mediated by a number of cytokines (reviewed in<sup>[82]</sup>), the majority of which ultimately act on two key transcription factors: peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) and Runx2, the master regulators of adipogenesis and osteogenesis, respectively.

In addition to its regulation of adipogenesis, PPAR $\gamma$  plays another role in osteoblast and osteoclast function, making it a viable target for bone tissue regeneration. In a recent study, James *et al* demonstrated that lentiviral delivery of PPAR $\gamma$  shRNA led to increased trabecular bone volume, thickness, and number in the femoral bone of mice<sup>[81]</sup>. Specifically, PPAR $\gamma$  shRNA significantly upregulated the mRNA expression of osteogenic gene markers (Runx2, Osteocalcin, and Alkaline phosphatase) while downregulating osteoclast marker activity in BMSCs. Interestingly, however, cortical bone thickness remained unaffected, and treatment resulted in significant reduction of intra-marrow lipid content *in vitro* and decreased mature marrow adipocytes *in vivo*, accompanied by reduced adipocyte-specific markers. Other investigators have gone on to show that increased expression of PPAR $\gamma$  is required to convert immature osteoblasts into adipocytes<sup>[83,84]</sup>. Likewise, gain in PPAR $\gamma$  function promotes the osteoclast lineage commitment and maturation process. Conversely, suppression or haploinsufficiency of PPAR $\gamma$  stimulates osteoblastogenesis and bone mass increase while inhibiting adipogenesis, osteoclastogenesis, and bone resorption<sup>[85-87]</sup>. Taken together, lentiviral delivery of PPAR $\gamma$  shRNA represents a promising technique to induce anabolic effects via trabecular bone formation, all while simultaneously downregulating adipogenic effects.

More predictable, however, is the osteogenic role of Runx2, which is generally regarded as the principal regulator of osteogenesis. In one study, Lee *et al* used a bicistronic vector encoding BMP2 in combination with the Runx2 gene to evaluate ASCs for osteogenic potential<sup>[48]</sup>. Transfected ASCs showed a marked improvement in bone formation compared to control; increase in alkaline phosphatase activity was found in the former, while PCR analysis and alizarin red staining revealed increased mineralization and high expression of osteogenesis-related markers, namely Opn, osteocalcin, and Collagen I. Similarly, gene therapy using Runx2 alone to transfect ASCs have been shown to also be successful in an osteoporotic mandibular distraction osteoporosis model in rabbits<sup>[88]</sup>. It is worth noting that

the current strategies utilizing ASCs now take advantage of the previously described inverse relationship between adipogenic and osteogenic differentiation, namely through upregulation of cytokines that are simultaneously pro-osteogenic and anti-adipogenic, as opposed to BMP2 which generally upregulates both<sup>[76]</sup>. One such potent osteogenic factor is NEL-like molecule (NELL1); in a study by Liu *et al*, ASCs were transfected with NELL1 through use of lentiviral vector<sup>[89]</sup>. Interestingly, in comparison to BMP2 control, the NELL-1 treatment group dramatically inhibited adipogenic differentiation while still retaining upregulated expression of osteogenic markers. Taken together, these studies suggest that gene therapy can effectively be utilized to redirect adipose stem cells towards an osteoblastic lineage.

### Targeting Endothelium

Another attractive target for bone tissue regeneration is endothelial cells, which is reasonable considering the high amount of vascularization required for sufficient bone growth. Interestingly, conventional bone tissue engineering techniques often overlook, and is devoid of, vasculature, which is critical in the transport of oxygen, nutrients, and soluble factors among other variables<sup>[90]</sup>. Perhaps the most well-understood angiogenic factor is vascular endothelial growth factor (VEGF). As a highly specific mitogen for endothelial cells, VEGF signal transduction results in endothelial cell proliferation, migration, and new vessel formation<sup>[91-98]</sup>. In addition to its role in angiogenesis, VEGF was found to help direct ASC differentiation towards an osteogenic lineage in ASCs<sup>[99]</sup>. Likewise, Jacobsen *et al* recently demonstrated in a murine tibia distraction osteogenesis model that both VEGFR1 and VEGFR2 are necessary to promote osteogenic lineage progression; inhibition via antibody blockade of one and/or both receptors resulted in decreased bone formation<sup>[100]</sup>. Reumann *et al* also confirmed the role of VEGFR1 and VEGFR2 in angiogenesis during bone repair in a mouse rib fracture model<sup>[101]</sup>; production of VEGF receptor 1 and 2 mRNA and protein is differential during endochondral bone repair. Specifically, VEGFR2 peaked at callous maturation. Furthermore, inhibition of VEGF synthesis and function via antisense oligonucleotide and suramin counteracted BMP-7-induced alkaline phosphatase activity and bone nodule formation<sup>[102]</sup>. Conversely, the application of VEGF with recombinant bone morphogenetic protein-2 (rhBMP-2) both *in vitro*, using human MSCs, and *in vivo*, using rats with calvarial defects, showed synergistic osteoinduction over BMP-2 alone, as measured by increased trabecular number<sup>[103]</sup>. Future studies analyzing if the endothelial cells only enhance the osteogenic niche by proving a vascular network for early osteoblasts and subsequent osteocytes are needed. Studies also have shown that these endothelial cells can undergo endothelial to mesenchymal transition which subsequently can undergo osteogenic differentiation. Taken together, these findings are indicative of the potential use of VEGF in bone tissue engineering for regenerative and therapeutic applications.

Similar to VEGF, fibroblast growth factor (FGF)-2 has also been shown to enhance bone formation. Compared to treatment with just rhBMP-2, addition of FGF led to increased bone surface ratio and significantly increased trabecular thickness in rats with calvarial defects<sup>[103]</sup>. Shinozaki *et al* also showed that FGF-2 played a significant role in bone formation as it induces genes such as *Collagen 1*, *Runx2*, *Osx*, and *Opn*, which are all involved in the initial stages of osteogenesis<sup>[104]</sup>. Rats with calvarial defects that were injected with DNA/protamine complex paste (D/P) and FGF-2 showed superior bone formation; the control, D/P, and D/P with FGF-2 rats showed 6.7%, 58.3%, and 67.0% newly formed bone

respectively after three months. Taken together, targeting angiogenic growth via epithelium development represents a viable strategy towards targeting bone growth indirectly. Thus, with studies showing its role in upregulating pro-osteogenic genes, FGF-2 shows potential as another delivery product for genetic bone tissue engineering.

While vital for angiogenesis, VEGF and FGF alone are not sufficient for a functional vasculature<sup>[105]</sup>. Rather than targeting a single agent, stimulating angiogenesis would be more efficacious by targeting an upstream agent which controls various other angiogenic genes<sup>[106]</sup>. One such popular upstream target is hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), which regulates many angiogenic key genes including *VEGF*, *TGF- $\beta$* , *PLGF*, *ANGPT1*, *SCF*, *SDF-1*, and platelet-derived growth factor (*PDGF*)-*B*<sup>[107,108]</sup>. Under hypoxic conditions, stabilized HIF-1 $\alpha$  translocates to the nucleus, where it dimerizes with HIF-1 $\beta$  and binds to a hypoxia response element (HRE) present on multiple hypoxia-related genes for angiogenic cytokines, (VEGF, bFGF, PDGF-B and angiopoietin-2), that are essential for endothelial cell motility, recruitment and proliferation<sup>[6]</sup>. HIF-1 $\alpha$  signaling is key to sustain differentiation of hypoxic prechondrogenic cells during skeletogenesis by regulating sex determining region Y-box 9 (SOX-9) which is required for the cartilage precursor to HO. Furthermore, hypoxia is known to up-regulate BMP2 in osteoblasts through activation of HIF-1 $\alpha$  signaling<sup>[109]</sup>. Studies have shown that HIF-1 $\alpha$  overexpression results in upregulation of VEGF and SDF-1 expression in BMSCs<sup>[110]</sup>. Likewise, the constitutively active form of HIF-1 $\alpha$  (CA5), transduced to BMSCs via lentivirus vector, upregulates a number of angiogenic factors downstream associated with the process of angiogenesis in rat calvarial defect models<sup>[111]</sup>; these factors include VEGF, SDF-1, bFGF, PLGF, ANGPT1, and SCF, both at the mRNA and protein level. Furthermore, immunohistochemistry showed increased HIF-1 $\alpha$  expression in the treatment groups in bone matrix and surrounding fibroblastic-like tissue, whereas the control groups showed no obvious staining. Likewise, the HIF-1 $\alpha$  group was shown to have significantly larger areas of newly formed blood vessels in the model system<sup>[111]</sup>. Thus, bone tissue regeneration via targeting of upstream angiogenic genes, namely *HIF-1 $\alpha$* , represents a promising alternative approach utilizing supporting tissue.

#### Targeting Skeletal Muscle Cells

As well, it is possible to influence muscle tissue to differentiate into bone using the described viral-based genetic engineering techniques, most frequently through utilizing the BMP signaling pathway in myoblast precursor cells<sup>[73]</sup>. Specifically, investigators have thus far demonstrated that it is possible to differentiate the mouse myoblast cell line, C2C12, into an osteoblastic phenotype *in vitro*<sup>[112,113]</sup>. One study utilized truncated LIM mineralization protein-1 (LMP-1), which is an intracellular osteoinductive protein, to induce bone formation<sup>[113]</sup>. Overexpression of truncated human LMP-1 [hLMP-1(t)] demonstrated an inhibitory effect on myotube formation in C2C12 cultures, while alkaline phosphatase activity was significantly enhanced compared to control cultures. Likewise, Osteocalcin, BMP-2, and BMP-7 expression also increased, all of which are markers of osteoblastic differentiation. Thus, unlike C2C12 cells in the control groups that went on to assume the myoblast phenotype, those transduced with hLMP-1(t) differentiated into osteoblastic cells<sup>[113]</sup>. In a similar study, instead of LMP-1, C2C12 cells were transfected with suppressor of cytokine signaling (SOCS)-2 to interfere with myotube formation, and upregulate both BMP and JunB expression in order to redirect towards an osteoblastic lineage<sup>[112]</sup>. Another comparable study utilizing the C2C12 cell line observed increased extracellular matrix

calcification, as measured by increased Alkaline phosphatase activity, with induction of both BMP-2 and retinoic acid<sup>[114]</sup>. In another study, Pola *et al* demonstrated that gene therapy as mediated via plasmid and adenoviral transfer to human LMP-1 induces expression of genes such as BMPs, which are involved in bone formation, and promote *in vitro* bone nodule formation in animal models via posterior thoracic and lumbar spine fusion<sup>[115]</sup>. LMP-3 induced expression of BMP-2, OSX, RunX2, and alkaline phosphatase in human MSCs, and direct gene transfer of hLMP-3 into murine skeletal muscle led to more efficient ectopic bone formation compared to BMP-2 treatment alone. As seen in bone-related pathological conditions, muscle cells are often exposed to increased levels of BMP, which are strong promoters of bone induction as well as inhibitors of myogenesis. For example, *Osterix* gene, a transcription factor for osteoblastogenesis, is upregulated with BMP stimulation in C2C12 cells<sup>[116]</sup>; thus, inhibition of Osterix should discourage osteoblastogenesis in myoblasts, which has been confirmed by *Pitx2* overexpression. In summary, there is strong evidence for the redirection of a myogenic lineage towards an osteoblastic one, most commonly through utilizing the BMP signaling pathway and downstream Osterix transcription factor.

## CONCLUSIONS

In summary, gene therapy shows significant potential in bone regenerative applications. Investigators have implemented a variety of delivery systems including retroviral, lentiviral, adenoviral, and siRNA to either replace or silence defective genes to restore gene function. There are also several alternative non-integrating methods, the most popular of which are minicircles, as well as various products for delivery, ranging from transcription factors and cytokines to miRNA. Taken together, there is a wide variety of combinations of vectors and delivery products that are still undergoing investigation. Rather than targeting bone tissue directly, investigators have also explored the manner in which targeting bone supporting tissues influences bone formation. This includes the targeting of resident multilineage progenitor cells or even the redirection/transdifferentiation of mature adipocytes or skeletal muscle cells to the osteoblastic lineage. In summary, with an increasing interest in the development of viable alternatives to traditional bone grafts, gene therapy for bone tissue engineering and regeneration is a rapidly increased and promising field.

## CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

## REFERENCES

- 1 Polo-Corrales L, Latorre-Esteves M, Ramirez-Vick JE. Scaffold design for bone regeneration. *J Nanosci Nanotechnol* 2014; **14**: 15-56
- 2 Giatsidis G, Dalla Venezia E, Bassetto F. The role of gene therapy in regenerative surgery: updated insights. *Plast Reconstr Surg* 2013; **131**: 1425-1435
- 3 Kofron MD, Laurencin CT. Bone tissue engineering by gene delivery. *Adv Drug Deliv Rev* 2006; **58**: 555-576
- 4 Jakob F, Ebert R, Ignatius A, Matsushita T, Watanabe Y, Groll J, Walles H. Bone tissue engineering in osteoporosis. *Maturitas* 2013; **75**: 118-124
- 5 Goessler UR, Riedel K, Hormann K, Riedel F. Perspectives of gene therapy in stem cell tissue engineering. *Cells Tissues Organs* 2006; **183**: 169-179
- 6 Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen

- BR. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med* 2010; **16**: 1400-1406
- 7 Heyde M, Partridge KA, Oreffo RO, Howdle SM, Shakesheff KM, Garnett MC. Gene therapy used for tissue engineering applications. *J Pharm Pharmacol* 2007; **59**: 329-350
- 8 Komesu MC, Lopes RA, Petenunci SO, Silva-Netto CR, Paula Lopes OV, Maia Campos G. [Morphological changes in the submandibular gland in aging rats]. *Rev Fac Odontol Ribeiro Preto* 1986; **23**: 79-88
- 9 Anson DS. The use of retroviral vectors for gene therapy-what are the risks? A review of retroviral pathogenesis and its relevance to retroviral vector-mediated gene delivery. *Genet Vaccines Ther* 2004; **2**: 9
- 10 Mollnes TE, Videm V, Riesenfeld J, Garred P, Svennevig JL, Fosse E, Hogasen K, Harboe M. Complement activation and bioincompatibility. The terminal complement complex for evaluation and surface modification with heparin for improvement of biomaterials. *Clin Exp Immunol* 1991; **86 Suppl 1**: 21-26
- 11 Matsuoka M. Human T-cell leukemia virus type I and adult T-cell leukemia. *Oncogene* 2003; **22**: 5131-5140
- 12 Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nussbaum P, Selz F, Hue C, Certain S, Casanova JL, Bousso P, Deist FL, Fischer A. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science* 2000; **288**: 669-672
- 13 Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, Radford I, Villeval JL, Fraser CC, Cavazzana-Calvo M, Fischer A. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. *N Engl J Med* 2003; **348**: 255-256
- 14 Bosticardo M, Ferrua F, Cavazzana M, Aiuti A. Gene therapy for Wiskott-Aldrich Syndrome. *Curr Gene Ther* 2014
- 15 Qasim W, Gennery AR. Gene therapy for primary immunodeficiencies: current status and future prospects. *Drugs* 2014; **74**: 963-969
- 16 Kafri T. Gene delivery by lentivirus vectors an overview. *Methods Mol Biol* 2004; **246**: 367-390
- 17 Cockrell AS, Kafri T. Gene delivery by lentivirus vectors. *Molecular Biotechnol* 2007; **36**: 184-204
- 18 Jaako P, Debnath S, Olsson K, Modlich U, Rothe M, Schambach A, Flygare J, Karlsson S. Gene therapy cures the anemia and lethal bone marrow failure in mouse model for RPS19-deficient Diamond-Blackfan anemia. *Haematologica* 2014
- 19 Betchen SA, Kaplitt M. Future and current surgical therapies in Parkinson's disease. *Current Opin Neurol* 2003; **16**: 487-493
- 20 Federici T, Kutner R, Zhang XY, Kuroda H, Tordo N, Boulis NM, Reiser J. Comparative analysis of HIV-1-based lentiviral vectors bearing lyssavirus glycoproteins for neuronal gene transfer. *Genet Vaccines Ther* 2009; **7**: 1
- 21 Kantor B, McCown T, Leone P, Gray SJ. Clinical applications involving CNS gene transfer. *Adv Genet* 2014; **87**: 71-124
- 22 Bett AJ, Prevec L, Graham FL. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 1993; **67**: 5911-5921
- 23 Vorburger SA, Hunt KK. Adenoviral gene therapy. *Oncologist* 2002; **7**: 46-59
- 24 Schiedner G, Morral N, Parks RJ, Wu Y, Koopmans SC, Langston C, Graham FL, Beaudet AL, Kochanek S. Genomic DNA transfer with a high-capacity adenovirus vector results in improved *in vivo* gene expression and decreased toxicity. *Nat Genet* 1998; **18**: 180-183
- 25 Hara K, Kageji T, Mizobuchi Y, Kitazato KT, Okazaki T, Fujihara T, Nakajima K, Mure H, Kuwayama K, Hara T, Nagahiro S. Blocking of the interaction between Wnt proteins and their co-receptors contributes to the anti-tumor effects of adenovirus-mediated DKK3 in glioblastoma. *Cancer Lett* 2014; **356(2)**: 496-505
- 26 Perez-Cruet MJ, Trask TW, Chen SH, Goodman JC, Woo SL, Grossman RG, Shine HD. Adenovirus-mediated gene therapy of experimental gliomas. *J Neurosci Res* 1994; **39**: 506-511
- 27 Mizuno M, Yoshida J, Colosi P, Kurtzman G. Adeno-associated virus vector containing the herpes simplex virus thymidine kinase gene causes complete regression of intracerebrally implanted human gliomas in mice, in conjunction with ganciclovir administration. *Jpn J Cancer Res* 1998; **89**: 76-80
- 28 Alden TD, Pittman DD, Hankins GR, Beres EJ, Engh JA, Das S, Hudson SB, Kerns KM, Kallmes DF, Helm GA. *In vivo* endochondral bone formation using a bone morphogenetic protein 2 adenoviral vector. *Hum Gene Ther* 1999; **10**: 2245-2253
- 29 Baltzer AW, Lattermann C, Whalen JD, Wooley P, Weiss K, Grimm M, Ghivizzani SC, Robbins PD, Evans CH. Genetic enhancement of fracture repair: healing of an experimental segmental defect by adenoviral transfer of the BMP-2 gene. *Gene Ther* 2000; **7**: 734-739
- 30 Kang Q, Sun MH, Cheng H, Peng Y, Montag AG, Deyrup AT, Jiang W, Luu HH, Luo J, Szatkowski JP, Vanichakarn P, Park JY, Li Y, Haydon RC, He TC. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. *Gene Ther* 2004; **11**: 1312-1320
- 31 Gary DJ, Puri N, Won YY. Polymer-based siRNA delivery: perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. *J Control Release* 2007; **121**: 64-73
- 32 Bartlett DW, Davis ME. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res* 2006; **34**: 322-333
33. Hoelters J, Ciccarella M, Drechsel M, Geissler C, Gulkan H, Bocker W, Schieker M, Jochum M, Neth P. Nonviral genetic modification mediates effective transgene expression and functional RNA interference in human mesenchymal stem cells. *J Gene Med* 2005; **7**: 718-728
- 34 Zou GM. RNAi in stem cells: current status and future perspectives. *Methods Mol Biol* 2010; **650**: 3-14
- 35 Zhang Y, Wei L, Miron RJ, Zhang Q, Bian Z. Prevention of Alveolar Bone Loss in an Osteoporotic Animal Model via Interference of Semaphorin 4d. *J Dent Res* 2014; **93**: 1095-1100
- 36 Benihoud K, Yeh P, Perriacaudet M. Adenovirus vectors for gene delivery. *Curr Opin Biotechnol* 1999; **10**: 440-447
- 37 Romano G, Marino IR, Pentimalli F, Adamo V, Giordano A. Insertional mutagenesis and development of malignancies induced by integrating gene delivery systems: implications for the design of safer gene-based interventions in patients. *Drug News Perspect* 2009; **22**: 185-196
- 38 Jia F, Wilson KD, Sun N, Gupta DM, Huang M, Li Z, Panetta NJ, Chen ZY, Robbins RC, Kay MA, Longaker MT, Wu JC. A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 2010; **7**: 197-199
- 39 Levi B, Hyun JS, Nelson ER, Li S, Montoro DT, Wan DC, Jia FJ, Glotzbach JC, James AW, Lee M, Huang M, Quarto N, Gurtner GC, Wu JC, Longaker MT. Nonintegrating knockdown and customized scaffold design enhances human adipose-derived stem cells in skeletal repair. *Stem Cells* 2011; **29**: 2018-2029
- 40 Stenler S, Blomberg P, Smith CE. Safety and efficacy of DNA vaccines: Plasmids vs minicircles. *Human Vaccin Immunother* 2014; **10(5)**. DOI: 10.4161/hv.28077
- 41 Osawa K, Okubo Y, Nakao K, Koyama N, Bessho K. Osteoinduction by microbubble-enhanced transcutaneous sonoporation of human bone morphogenetic protein-2. *J Gene Med* 2009; **11**: 633-641
- 42 Nishida K, Doita M, Takada T, Kakutani K, Miyamoto H, Shimomura T, Maeno K, Kurosaka M. Sustained transgene expression in intervertebral disc cells *in vivo* mediated by microbubble-enhanced ultrasound gene therapy. *Spine* 2006; **31**: 1415-1419

- 43 Haleem-Smith H, Derfoul A, Okafor C, Tuli R, Olsen D, Hall DJ, Tuan RS. Optimization of high-efficiency transfection of adult human mesenchymal stem cells *in vitro*. *Molecular Biotechnol* 2005; **30**: 9-20
- 44 Lakshminpathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS, Verfaillie CM. Efficient transfection of embryonic and adult stem cells. *Stem Cells* 2004; **22**: 531-543
- 45 Pensak MJ, Lieberman JR. Gene therapy for bone regeneration. *Curr Pharm Des* 2013; **19**: 3466-3473
- 46 Im GI. Nonviral gene transfer strategies to promote bone regeneration. *J Biomed Mater Res A* 2013; **101**: 3009-3018
- 47 Lee JS, Lee JM, Im GI. Electroporation-mediated transfer of Runx2 and Osterix genes to enhance osteogenesis of adipose stem cells. *Biomaterials* 2011; **32**: 760-768
- 48 Lee SJ, Kang SW, Do HJ, Han I, Shin DA, Kim JH, Lee SH. Enhancement of bone regeneration by gene delivery of BMP2/Runx2 bicistronic vector into adipose-derived stromal cells. *Biomaterials* 2010; **31**: 5652-5659
- 49 Park J, Ries J, Gelse K, Kloss F, von der Mark K, Wiltfang J, Neukam FW, Schneider H. Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. *Gene Ther* 2003; **10**: 1089-1098
- 50 Lutz R, Park J, Felszeghy E, Wiltfang J, Nkenke E, Schlegel KA. Bone regeneration after topical BMP-2-gene delivery in circumferential peri-implant bone defects. *Clin Oral Implants Res* 2008; **19**: 590-599
- 51 Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. *Science* 2001; **294**: 862-864
- 52 Ambros V. The functions of animal microRNAs. *Nature* 2004; **431**: 350-355
- 53 Eskildsen T, Taipaleenmaki H, Stenvang J, Abdallah BM, Ditzel N, Nossent AY, Bak M, Kauppinen S, Kassem M. MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells *in vivo*. *Proc Natl Acad Sci U S A* 2011; **108**: 6139-6144
- 54 Huang J, Zhao L, Xing L, Chen D. MicroRNA-204 regulates Runx2 protein expression and mesenchymal progenitor cell differentiation. *Stem Cells* 2010; **28**: 357-364
- 55 Wu T, Zhou H, Hong Y, Li J, Jiang X, Huang H. miR-30 family members negatively regulate osteoblast differentiation. *J Biol Chem* 2012; **287**: 7503-7511
- 56 Oskowitz AZ, Lu J, Penfornis P, Ylostalo J, McBride J, Flemington EK, Prockop DJ, Pochampally R. Human multipotent stromal cells from bone marrow and microRNA: regulation of differentiation and leukemia inhibitory factor expression. *Proc Natl Acad Sci U S A* 2008; **105**: 18372-18377
- 57 Li H, Xie H, Liu W, Hu R, Huang B, Tan YF, Xu K, Sheng ZF, Zhou HD, Wu XP, Luo XH. A novel microRNA targeting HDAC5 regulates osteoblast differentiation in mice and contributes to primary osteoporosis in humans. *J Clin Invest* 2009; **119**: 3666-3677
- 58 Li Z, Hassan MQ, Jafferji M, Aqeilan RI, Garzon R, Croce CM, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. *J Biol Chem* 2009; **284**: 15676-15684
- 59 Kim YJ, Bae SW, Yu SS, Bae YC, Jung JS. miR-196a regulates proliferation and osteogenic differentiation in mesenchymal stem cells derived from human adipose tissue. *J Bone Miner Res* 2009; **24**: 816-825
- 60 Li H, Li T, Wang S, Wei J, Fan J, Li J, Han Q, Liao L, Shao C, Zhao RC. miR-17-5p and miR-106a are involved in the balance between osteogenic and adipogenic differentiation of adipose-derived mesenchymal stem cells. *Stem Cell Res* 2013; **10**: 313-324
- 61 Kapinas K, Kessler C, Ricks T, Gronowicz G, Delany AM. miR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. *J Biol Chem* 2010; **285**: 25221-25231
- 62 Deng Y, Zhou H, Zou D, Xie Q, Bi X, Gu P, Fan X. The role of miR-31-modified adipose tissue-derived stem cells in repairing rat critical-sized calvarial defects. *Biomaterials* 2013; **34**: 6717-6728
- 63 Gao J, Yang T, Han J, Yan K, Qiu X, Zhou Y, Fan Q, Ma B. MicroRNA expression during osteogenic differentiation of human multipotent mesenchymal stromal cells from bone marrow. *J Cell Biochem* 2011; **112**: 1844-1856
- 64 Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**: 143-147
- 65 Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. *Exp Biol Med* 2001; **226**: 507-520
- 66 Gottschling S, Saffrich R, Seckinger A, Krause U, Horsch K, Miesala K, Ho AD. Human mesenchymal stromal cells regulate initial self-renewing divisions of hematopoietic progenitor cells by a beta1-integrin-dependent mechanism. *Stem Cells* 2007; **25**: 798-806
- 67 Chen CP, Lee MY, Huang JP, Aplin JD, Wu YH, Hu CS, Chen PC, Li H, Hwang SM, Liu SH, Yang YC. Trafficking of multipotent mesenchymal stromal cells from maternal circulation through the placenta involves vascular endothelial growth factor receptor-1 and integrins. *Stem Cells* 2008; **26**: 550-561
- 68 Tokunaga A, Oya T, Ishii Y, Motomura H, Nakamura C, Ishizawa S, Fujimori T, Nabeshima Y, Umezawa A, Kanamori M, Kimura T, Sasahara M. PDGF receptor beta is a potent regulator of mesenchymal stromal cell function. *J Bone Miner Res* 2008; **23**: 1519-1528
- 69 Wagner W, Wein F, Roderburg C, Saffrich R, Diehlmann A, Eckstein V, Ho AD. Adhesion of human hematopoietic progenitor cells to mesenchymal stromal cells involves CD44. *Cells Tissues Organs* 2008; **188**: 160-169
- 70 Wozney JM. Overview of bone morphogenetic proteins. *Spine* 2002; **27**: S2-8
- 71 Cook SD, Salkeld SL, Brinker MR, Wolfe MW, Rueger DC. Use of an osteoinductive biomaterial (rhOP-1) in healing large segmental bone defects. *J Orthop Trauma* 1998; **12**: 407-412
- 72 Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC, Whitecloud TS. The effect of recombinant human osteogenic protein-1 on healing of large segmental bone defects. *J Bone Joint Surg Am* 1994; **76**: 827-838
- 73 Phillips JE, Gersbach CA, Garcia AJ. Virus-based gene therapy strategies for bone regeneration. *Biomaterials* 2007; **28**: 211-229
- 74 Baltzer AW, Lattermann C, Whalen JD, Ghivizzani S, Wooley P, Krauspe R, Robbins PD, Evans CH. Potential role of direct adenoviral gene transfer in enhancing fracture repair. *Clin Orthop Relat Res* 2000; **S120-125**
- 75 Chang SC, Lin TM, Chung HY, Chen PK, Lin FH, Lou J, Jeng LB. Large-scale bicortical skull bone regeneration using ex vivo replication-defective adenoviral-mediated bone morphogenetic protein-2 gene-transferred bone marrow stromal cells and composite biomaterials. *Neurosurgery* 2009; **65**: 75-81; discussion 81-73
- 76 James AW, Pang S, Askarinam A, Corselli M, Zara JN, Goyal R, Chang L, Pan A, Shen J, Yuan W, Stoker D, Zhang X, Adams JS, Ting K, Soo C. Additive effects of sonic hedgehog and Nell-1 signaling in osteogenic versus adipogenic differentiation of human adipose-derived stromal cells. *Stem Cells Dev* 2012; **21**: 2170-2178
- 77 Ye ZC, Zhang WB, Chen XL, Chen J, Li ZL, Ke SN. [Maxillary sinus floor augmentation using gene-enhanced tissue engineered bone]. *Zhonghua Kou Qiang Yi Xue Za Zhi* 2012; **47**: 114-117
- 78 Xu XL, Tang T, Dai K, Zhu Z, Guo XE, Yu C, Lou J. Immune response and effect of adenovirus-mediated human BMP-2 gene transfer on the repair of segmental tibial bone defects in goats. *Acta Orthop* 2005; **76**: 637-646

- 79 Musgrave DS, Bosch P, Ghivizzani S, Robbins PD, Evans CH, Huard J. Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone* 1999; **24**: 541-547
- 80 Oryan A, Alidadi S, Moshiri A, Bigham-Sadegh A. Bone morphogenetic proteins: A powerful osteoinductive compound with non-negligible side effects and limitations. *BioFactors* 2014
- 81 James AW, Shen J, Khadarian KA, Pang S, Chung G, Goyal R, Asatrian G, Velasco O, Kim J, Zhang X, Ting K, Soo C. Lentiviral delivery of PPARgamma shRNA Alters the Balance of Osteogenesis and Adipogenesis, Improving Bone Microarchitecture. *Tissue Eng Part A* 2014
- 82 James AW. Review of Signaling Pathways Governing MSC Osteogenic and Adipogenic Differentiation. *Scientifica* 2013; **2013**: 684736
- 83 Kim SW, Her SJ, Kim SY, Shin CS. Ectopic overexpression of adipogenic transcription factors induces transdifferentiation of MC3T3-E1 osteoblasts. *Biochem Biophys Res Comm* 2005; **327**: 811-819
- 84 Yoshiko Y, Oizumi K, Hasegawa T, Minamizaki T, Tanne K, Maeda N, Aubin JE. A subset of osteoblasts expressing high endogenous levels of PPARgamma switches fate to adipocytes in the rat calvaria cell culture model. *PLoS One* 2010; **5**: e11782
- 85 Takada I, Suzawa M, Matsumoto K, Kato S. Suppression of PPAR transactivation switches cell fate of bone marrow stem cells from adipocytes into osteoblasts. *Ann N Y Acad Sci* 2007; **1116**: 182-195
- 86 Kawaguchi H, Akune T, Yamaguchi M, Ohba S, Ogata N, Chung UI, Kubota N, Terauchi Y, Kadowaki T, Nakamura K. Distinct effects of PPARgamma insufficiency on bone marrow cells, osteoblasts, and osteoclastic cells. *J Bone Miner Metab* 2005; **23**: 275-279
- 87 Wan Y, Chong LW, Evans RM. PPAR-gamma regulates osteoclastogenesis in mice. *Nat Med* 2007; **13**: 1496-1503
- 88 Sun JJ, Zheng XH, Wang LY, Liu L, Jing W, Lin YF, Tian W, Tang W, Long J. New bone formation enhanced by ADSCs overexpressing hRunx2 during mandibular distraction osteogenesis in osteoporotic rabbits. *J Orthop Res* 2014; **32**: 709-720
- 89 Liu Y, Chen C, He H, Wang D, E L, Liu Z, Liu H. Lentiviral-mediated gene transfer into human adipose-derived stem cells: role of NELL1 versus BMP2 in osteogenesis and adipogenesis *in vitro*. *Acta Biochim Biophys Sin* 2012; **44**: 856-865
- 90 Santos MI, Reis RL. Vascularization in bone tissue engineering: physiology, current strategies, major hurdles and future challenges. *Macromol Biosci* 2010; **10**: 12-27
- 91 Hoeben A, Landuyt B, Highley MS, Wildiers H, Van Oosterom AT, De Bruijn EA. Vascular endothelial growth factor and angiogenesis. *Pharmacol Rev* 2004; **56**: 549-580
- 92 Ortega N, Hutchings H, Plouet J. Signal relays in the VEGF system. *Front Biosci* 1999; **4**: D141-152
- 93 Joukov V, Kaipainen A, Jeltsch M, Pajusola K, Olofsson B, Kumar V, Eriksson U, Alitalo K. Vascular endothelial growth factors VEGF-B and VEGF-C. *J Cell Physiol* 1997; **173**: 211-215
- 94 Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A* 1998; **95**: 548-553
- 95 Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M. A novel type of vascular endothelial growth factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. *J Biol Chem* 1998; **273**: 31273-31282
- 96 Andre T, Kotelevets L, Vaillant JC, Coudray AM, Weber L, Prevot S, Parc R, Gespach C, Chastre E. Vegf, Vegf-B, Vegf-C and their receptors KDR, FLT-1 and FLT-4 during the neoplastic progression of human colonic mucosa. *Int J Cancer* 2000; **86**: 174-181
- 97 Partanen TA, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG, Alitalo K. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J* 2000; **14**: 2087-2096
- 98 Bernatchez PN, Rollin S, Soker S, Sirois MG. Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration and PAF synthesis: Role of neuropilin-1. *J Cell Biochem* 2002; **85**: 629-639
- 99 Clark D, Wang X, Chang S, Czajka-Jakubowska A, Clarkson BH, Liu J. VEGF promotes osteogenic differentiation of ASCs on ordered fluorapatite surfaces. *J Biomed Mater Res A* 2014
- 100 Jacobsen KA, Al-Aql ZS, Wan C, Fitch JL, Stapleton SN, Mason ZD, Cole RM, Gilbert SR, Clemens TL, Morgan EF, Einhorn TA, Gerstenfeld LC. Bone formation during distraction osteogenesis is dependent on both VEGFR1 and VEGFR2 signaling. *J Bone Miner Res* 2008; **23**: 596-609
- 101 Reumann MK, Nair T, Strachna O, Boskey AL, Mayer-Kuckuk P. Production of VEGF receptor 1 and 2 mRNA and protein during endochondral bone repair is differential and healing phase specific. *J Appl Physiol* 2010; **109**: 1930-1938
- 102 Yeh LC, Lee JC. Osteogenic protein-1 increases gene expression of vascular endothelial growth factor in primary cultures of fetal rat calvaria cells. *Mol Cell Endocrinol* 1999; **153**: 113-124
- 103 Lee JH, Jang SJ, Baek HR, Lee KM, Chang BS, Lee CK. Synergistic induction of early stage of bone formation by combination of recombinant human bone morphogenetic protein-2 and epidermal growth factor. *J Tissue Eng Regen Med* 2014
- 104 Shinozaki Y, Toda M, Ohno J, Kawaguchi M, Kido H, Fukushima T. Evaluation of bone formation guided by DNA/protamine complex with FGF-2 in an adult rat calvarial defect model. *J Biomed Mater Res B Appl Biomater* 2014
- 105 Carmeliet P. VEGF gene therapy: stimulating angiogenesis or angioma-genesis? *Nat Med* 2000; **6**: 1102-1103
- 106 Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nat Med* 2000; **6**: 460-463
- 107 Madeddu P. Therapeutic angiogenesis and vasculogenesis for tissue regeneration. *Exp Physiol* 2005; **90**: 315-326
- 108 Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 2003; **9**: 677-684
- 109 Amarilio R, Viukov SV, Sharir A, Eshkar-Oren I, Johnson RS, Zelzer E. HIF1alpha regulation of Sox9 is necessary to maintain differentiation of hypoxic prechondrogenic cells during early skeletogenesis. *Development* 2007; **134**: 3917-3928
- 110 Zou D, Zhang Z, Ye D, Tang A, Deng L, Han W, Zhao J, Wang S, Zhang W, Zhu C, Zhou J, He J, Wang Y, Xu F, Huang Y, Jiang X. Repair of critical-sized rat calvarial defects using genetically engineered bone marrow-derived mesenchymal stem cells overexpressing hypoxia-inducible factor-1alpha. *Stem Cells* 2011; **29**: 1380-1390
- 111 Zou D, Zhang Z, He J, Zhang K, Ye D, Han W, Zhou J, Wang Y, Li Q, Liu X, Zhang X, Wang S, Hu J, Zhu C, Zhang W, Zhou Y, Fu H, Huang Y, Jiang X. Blood vessel formation in the tissue-engineered bone with the constitutively active form of HIF-1alpha mediated BMSCs. *Biomaterials* 2012; **33**: 2097-2108
- 112 Ouyang X, Fujimoto M, Nakagawa R, Serada S, Tanaka T, Nomura S, Kawase I, Kishimoto T, Naka T. SOCS-2 interferes with myotube formation and potentiates osteoblast differentiation through upregulation of JunB in C2C12 cells. *J Cell Physiol* 2006; **207**: 428-436
- 113 Fei Q, Boden SD, Sangadala S, Viggswarapu M, Liu Y, Titus L. Truncated human LMP-1 triggers differentiation of C2C12 cells to an osteoblastic phenotype *in vitro*. *Acta Biochim Biophys Sin* 2007; **39**: 693-700

- 114 Karakida T, Yui R, Suzuki T, Fukae M, Oida S. Retinoic acid receptor gamma-dependent signaling cooperates with BMP2 to induce osteoblastic differentiation of C2C12 cells. *Connect Tiss Res* 2011; **52**: 365-372
- 115 Pola E, Gao W, Zhou Y, Pola R, Lattanzi W, Sfeir C, Gambotto A, Robbins PD. Efficient bone formation by gene transfer of human LIM mineralization protein-3. *Gene Ther* 2004; **11**: 683-693
- 116 Hayashi M, Maeda S, Aburatani H, Kitamura K, Miyoshi

H, Miyazono K, Imamura T. Pitx2 prevents osteoblastic transdifferentiation of myoblasts by bone morphogenetic proteins. *J Biol Chem* 2008; **283**: 565-571

**Peer reviewer:** Shirley Caroline Keolebogile Motaung, Assistant Dean: Postgraduate Studies, Research and Innovation, Faculty of Science, Tshwane University of Technology, Private Bad X680, Pretoria, South Africa