Gene therapy for \textit{in vivo} bone formation: recent advances

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\textbf{Abstract.} – Gene therapy has developed during the last two decades as a promising strategy for orthopaedics applications, since several different gene transfer techniques proved to be effective, both \textit{in vitro} and \textit{in vivo}, for the induction of bone formation.

Successful results have been achieved with gene-based bone healing strategies in several preclinical studies, using different animal models. New genes and new viral and non-viral vector constructs have been developed to reduce the risks and safety issues, widening the field of possible applications and improving the potential therapeutic effects.

We review the latest gene transfer technologies employed for \textit{in vivo} bone formation, focusing on the recently identified network of growth factors and genes involved in the modulation of the osteogenetic process and on the variety of vectors utilized for gene delivery.

\textbf{Key Words:}

Gene therapy, Bone formation, Gene transfer technologies.

\section*{Introduction}

Since its conceptual origin, gene therapy has evolved as a novel promising strategy for the treatment of several both genetic and non-genetic conditions. In particular, the therapeutical use of genes to induce tissue regeneration proved to be effective and its application has been proposed for different branches of medicine, including orthopaedics\textsuperscript{1}. In this regard gene therapy has been proposed as an extremely useful tool in several conditions in which new bone formation is needed, such as bone fractures, pseudoarthrosis of the spine, post-traumatic bone loss, revision of total joint arthroplasty and tumor resection. All these conditions present difficult clinical challenges for the orthopaedics, resulting often in insufficient surgical results with serious consequences in public health and social economic loss.

Several studies described possible orthopaedic applications of gene therapy, suggesting the options of different gene transfer approaches for the healing of bone, cartilage, intervertebral disc, tendons and ligaments\textsuperscript{1-3}.

In particular the usefulness of delivering therapeutic gene products into specific anatomical sites and the advantages and reliability of gene transfer approaches for orthopaedic applications has been emphasized by important evidences: first, the same procedures that are already used for traditional bone surgery can be applied for \textit{in situ} targeted gene transfer; second, neither a sustained and persisting, nor a systemic transgene expression are required for the bone healing process. Recently, new gene products have been described and new gene transfer strategies have been employed in several animal models. The most recent advances in the field of gene therapy for the induction of bone formation are reported in this review, with particular attention on the role of new gene products involved in the induction of osteogenesis.

\textbf{Genes and Osteogenesis}

The development of bone tissue occurs through the sequential activation of several genes, which are involved in a complex network, leading to the final event represented by the maturation of osteoblasts from mes-
enchimal precursor cells. Foremost regulators of this process are the Bone Morpho-
genetic Proteins (BMPs), mainly represented by the BMP-2, a member of the TGF-β super-
family, able to induce the osteogenic phenotype in both pluripotent and differenti-
ated non-osseous mesenchimal cells. The BMPs, have been widely employed for bone heal-
ing modes and, in particular, BMP-2 and BMP-7 (osteoogenic protein 1, OP-1) have been approved for limited clinical use. Both BMPs and their inhibitors, such as Noggin and Sclerostin, are expressed and co-
operate in a complex network during bone development and their importance in the process is suggested by the skeletal defects phenotypes resulting from loss-of-function mutations. The tissue-specific gene expression involved in the BMP-2 signaling pathway is controlled by specific transcription factors, acting downstream to BMP-2 and organized in macromolecular complexes within the nucleus, along with the chromatin remodeling of gene promoters.

Among the bone-specific transcription factors required for osteoblast differentia-
tion, two recently characterized gene products, Runt related transcription factor 2 (RunX2) and Osterix (Osx), accomplish a critical role during both intramembranous and endochondral bone formation. RunX2 regulates different stages of the process, through complex interactions with other transcription factors, including Smad proteins, and induces the expression of bone-specific genes, coding for osteoblastic markers such as Osteocalcin (OC), Bone Sialoprotein (BSP), Osteopontin (OP) and Collagen type 1 (ColI). Osx seems to induce osteoblast differentiation of mesenchimal precursors, but a clear elucidation of its specific function is still lacking.

Recently, Lim Mineralization Proteins (LMP), coded by three different splice vari-
ants (LMP-1, LMP-2, LMP-3) of the same gene, have been described to act as regulators of the osteoblast differentiation program. Different degrees of expression have been demonstrated for the three splice-variants of the LMP gene, by semi-quantitative RT-PCR on bone tissue from the iliac crest, correlating with the age of the tested individuals and suggesting a role of the LMPs in human bone metabolism. The secondary structure of the LMP includes highly conserved domains (PDZ and LIM) which mediate protein-protein interactions. The LIM domains of the protein, retained in LMP-1 and LMP-2 and lacking in LMP-3, are supposed not to be necessary for the osteogenic function. In particular, the osteoinductive properties of LMP-1, the main splice-variant, are exerted by the up-regulation of BMP-2, BMP-4, BMP-6, BMP-7 and TGF-β1, inducing direct membranous bone formation. This process includes an elevated production of proteoglycans, secondary to LMP-1 action on BMPs. In addiction, the LMP-3 splice variant, containing only the PDZ domain plus a “unique” region shared with LMP-1, is able to induce the expression of bone-specific genes (OC, OP, BSP) in both pre-osteoblastic and non-osteoblastic cell lines, along with in vitro mineralization and in vivo ectopic bone formation.

The function of several non-bone-specific proteins and growth factors, such as IGFs, FGF, TGF-β, VEGF, SHH, β-Catenin, Msx2 and the ubiquitin-proteasome machinery, have been reported as important mediators of osteoblast development.

The complete frame of pathways involved in the bone formation process is therefore a complex network of growth factors, intracel-
lar regulators and tissue-specific messengers, operating on the basis of an accurate regulation of their spatial and temporal expression.

**Gene Transfer Strategies for Bone Formation**

Several different approaches have been employed to deliver potential therapeutic genes into skeletal tissue for bone healing purposes. The most recurrent strategies are based on the application of viral vector-based gene transfer along with the use of cell-mediated gene transfer, though direct inoculation of recombinant genes (named “naked DNA”) and proteins proved to be also effective in inducing bone formation. Moreover, the efficiency of introduction of the therapeutic agents within the selected anatomical sites has been often improved using biological scaffolds, such as porous hydroxyapatite and collagen sponges.
Virus-Mediated Gene Transfer (Transduction)

Several virus species have been employed as vectors for gene delivery in order to induce bone formation. Viral vectors are characterized by a high efficiency of transduction, but many reasonable safety issues are still pending about their possible use in clinical application (Table I).

The viral species which proved to be more suitable as vectors for gene therapy in tissue repair models are Adenovirus (AdV) and Adeno-associated virus (AAV).

Tissue repair requires a short term expression of genes, initiating a cascade of events directing a self-maintained process, while a longer or even persisting action of the same factors could lead to excessive and abnormal bone formation. In this regard, the adenoviral vectors are the most suitable, being non-integrating and relatively safe viruses, inducing high level transient gene expression and allowing the transduction of several cell types and efficient delivery into muscle and connective tissue in vivo. In particular several groups have reported the successful use of replication-defective AdV vectors bearing recombinant human BMP-2 (rhBMP-2) for inducing bone formation in different animal models. Defective AdV vectors have been employed also for delivering other putative osteogenic genes including other members of the BMP family and the LMP splice variants, which proved to be effective in inducing bone formation in differentiated tissues and successful bone healing in bone defect models. An example of successful direct gene transfer using AdV vector carrying an rhBMP-2 gene for a non-union fracture model in immuno-competent rat (unpublished data) is shown in Figure 1.

While AdV vectors can induce transient gene expression, due to their non-integrated status within the cell genome, wild type AAV are able to integrate into cell chromosomes, though the insertional event is very rare in recombinant constructs, avoiding the insertional mutagenesis risks showed by retroviruses. This feature enables a long term transduction of cells, which usually is not desirable for bone healing purposes, although the gene expression can be temporally modulated by the integration of inducible promoters exogenously regulated. Few examples of

<table>
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<tr>
<th>Type of Vector</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Adenovirus</td>
<td>High efficiency of transduction</td>
<td>Frequent immunization in humans</td>
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<td></td>
<td>Wide tropism</td>
<td>High infectivity</td>
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<td></td>
<td>Ease of production, high titers</td>
<td>Short term transgene expression</td>
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<td>High capacity in gene packaging</td>
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<td>Adeno-associated</td>
<td>Safeness</td>
<td>Lower capacity in gene packaging</td>
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<td>Prolonged gene expression</td>
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<td>Retrovirus</td>
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<td>Plasmid</td>
<td>Safeness</td>
<td>Low efficiency</td>
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<td>Epicomic replication independent form the genome DNA</td>
<td>Barely suitable for in vivo applications</td>
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<tr>
<td>Liposomes, cationic lipids</td>
<td>Safeness</td>
<td>Very low efficiency</td>
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<td></td>
<td>Relatively high capacity in gene packaging</td>
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Table I. Main features of the principal vectors used for gene therapy in bone formation.
AAV constructs have been reported so far, delivering rhBMP-2\textsuperscript{77,78} and BMP-4\textsuperscript{79} for in vivo bone formation.

Oncoretroviruses (Rv), deriving from the Moloney Leukemia Virus (MLV), are RNA viruses able to integrate permanently in the host genome that have been widely employed for several ex vivo applications also in clinical studies which proved to be effective, though presenting serious secondary effects related to the insertional mutagenesis risk\textsuperscript{80}. Though their stable transduction properties do not match with the short term expression required for osteoinductive agents, great profit can be obtained from their peculiar multiplication features (i.e. they can reproduce only in dividing cells). In fact, Rv-vector constructs carrying a hybrid BMP-2/BMP-4 cDNA has been used to promote fracture healing in a rat model. The osteoinductive agent has been successful delivered selectively to the proliferating periosteal cells arising after bone fracture, with no evidence of external tissues transduction\textsuperscript{81}.

Another class of integrating viruses allowing stable transduction is the Lentivirus, specialized retrovirus deriving from the HIV, capable of random integration in the host cell genome, with consequent possible risks of insertional mutagenesis. For the same reasons above mentioned, the long-term gene expression is not an advantageous feature for bone healing application, thus lentivirus-based vectors have not been employed frequently for these applications and only a single group reported the successful use of this construct for delivering BMP-2 into cells, inducing long-term transgene expression and new bone formation in vivo\textsuperscript{82}.

### Non Viral Gene Transfer

Among the non-viral gene transfer vectors, plasmidic DNA have been largely employed, being able to trespass the cell membrane, by chemical transfection or electroporation techniques, and replicate independently from the genomic DNA. In particular, electroporation-based transfer of BMP-2 and BMP-4 has been employed for inducing bone formation in vivo\textsuperscript{83,84}. Though very low efficiency of transfection can be achieved by direct inoculation of plasmidic DNA, better results can be obtained implementing plasmid-mediated gene transfer with the use of biodegradable matrices and scaffolds for inducing bone formation in vivo\textsuperscript{85}.

Other chemical non-viral vectors for gene transfer are represented by cationic polymers and liposomes, which proved to be safe and effective when used for transfecting ex-vivo cells to be re-implanted in order to obtain bone healing in fracture models\textsuperscript{86,87}. Otherwise the association of liposomes or cationic lipids vectors with scaffolds or bone substitutes is necessary for treating bone defects in vivo\textsuperscript{88}.

### Cell-Mediated Gene Transfer

Promising results have been recently obtained by several groups reporting efficient bone formation in vivo by means of the introduction of genetically modified cells expressing genes for the different BMPs. Great advantages derive from the employment of engineered cells, which have been appropriately manipulated in vitro to express osteoinductive genes, since they can maintain physiologic doses of gene product for a sustained period once inoculated into a selected anatomical site, facilitating a more significant healing response\textsuperscript{89}. The cells used for this purpose are usually either adult stem cells, allowing heterologous transplantation even between different species, or differentiated cells obtained from the animal, ex vivo transplanted or transduced, then reinoculated, in order to induce bone formation in immunocompetent animals\textsuperscript{90,92}. Possible different effects resulting from the cell type used or the vector/gene transfer technique adopted.
have been evaluated in few studies, indicating that the AdVs are the more appropriate vectors for inducing the desired cell modification and that ex vivo strategies proved to be successful, unregarding the cell type used.

All these data suggest that defective viral vectors, mainly represented by AdV, are still considered the gold standard in gene therapy applications in the orthopaedic field, on the basis of the high level and short term transgene expression which they are able to mediate. Moreover, new promising evidences derive from cell-based therapy, which better resembles the biological environment.

In conclusion, great efforts have been dedicated in recent years to functional genomics studies, aimed at the identification of the molecular basis of many biological processes. This scientific progress led to the employment of gene therapy technologies to cure not only genetic diseases and consequently widened the possibilities of clinical applications. The successful preclinical results obtained with gene delivery into the injured bone tissue enables to consider gene therapy as a promising tool for the treatment of a wide range of skeletal defects, both genetic and environmental. Therefore, orthopaedics can now be considered one of the leading fields for application of gene therapy, suggesting and encouraging further improvements in the future.

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