GENE THERAPY FOR OSTEOPOROSIS

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ABSTRACT

Osteoporosis is considered one of the most common and serious problems affecting the elderly population worldwide. It is a chronic and progressive disease, characterized by decreased bone mass and degeneration of the microarchitecture of the bone tissue. Gene therapy represents a new approach in osteoporosis treatment, and its main function is to restore the compromised function in the metabolism. This review aims to elucidate the main studies on gene therapy in recent years, in the medical databases, that use gene therapy for the treatment of osteoporosis in animal models, as well as the future prospects of this therapy. The majority of the studies use the BMP, PTH and OPG genes, in an attempt to reestablish bone mass. Despite the lack of new molecules, all genes employed in these studies have proven to be efficient in the treatment of the disease. The benefits that gene therapy will provide for patients in the future should contribute substantially to increasing the quality of life for the elderly. Soon, clinical trials involving humans will benefit individuals with osteoporosis.

Keywords: *Osteoporosis. Gene therapy. Orthopaedics. Genetic vectors.*

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INTRODUCTION

Osteoporosis is considered a chronic and progressive disease, characterized by decreased bone mass and deterioration of the bone tissue microarchitecture. These changes lead to an increase of bone fragility and consequently, to a greater risk of fractures.[1]

Osteoporotic fractures are significant causes of functional loss and decrease in quality of life of the affected individual. The most accentuated loss of bone mass occurs in women from menopause, and is associated with estrogen insufficiency.[2]

The most severe fracture resulting from postmenopausal osteoporosis is that of the femur. In a study developed by Jiang *et al*.[3] in Canada, the mortality rate reached 6.3% in the first year and 30.8% in the second year after the fracture. This mortality rate is in conformity with the rest of the world, which varies from 1.02 to 10% during the hospital stay and from 23 to 30% during the first year after the fracture.[4]

In Brazil, the census figures draw attention to the large number of elderly individuals (14 million), the population with the greatest risk of developing osteoporosis. Over the period of one year 129,611 cases of osteoporosis were diagnosed in the private healthcare system, with an incidence of 4.99% in femoral fractures in the female population. The mean hospitalization time was higher than nine days and the direct cost per patient after hospitalization was calculated at R$ 24,000.00.[5]

In the city of São Paulo, a study with individuals over 65 years of age demonstrated that 92.8% of the women had osteopenia and/or osteoporosis in at least one part of the skeletal system analyzed.[6]

It is calculated that in 2050 there will be approximately 6.3 million fractures per year, with the majority occurring in Asia and in Latin America.[7]

Most of the drugs currently used for osteoporosis treatment have an antiresorptive (estrogens, raloxifene, calcium and vitamin D, alendronate, etidronate, risedronate and calcitonin) and/or anabolic (PTH 1-34 and strontium ranelate) action.[8]

Gene therapy has been indicated as a promising method for the treatment of osteometabolic diseases and represents a new approach in osteoporosis treatment. Gene therapy inserts the gene that codifies a protein which, if it were injected on its own, would be rapidly degraded.[9,10]

The basic principle of this therapy is to introduce exogenous genetic material to correct or modify cell function. Genetic material, called therapeutic gene, can be introduced into the target cell by genetic engineering techniques.[9,11]

The therapy starts with the modification of a basic structure, called plasmid. Plasmid is a small circular unit of DNA found in bacteria. Plasmid modification basically consists of the alteration of the expression cassette (promoter, complementary DNA of the therapeutic gene and a polyadenylation signal),

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which has the necessary regulatory signals for the expression of the gene of interest. The erroneous choice of any element may result in failure of the therapy or even be detrimental to the target cell.[11]

After the construction of the plasmid with the specific and necessary sequences for its maintenance and expression, this structure can be transferred to the target cell with the help of viral (transduction) and non-viral (transfection) vectors.[11,12] It is known that for the transduction of host cells, viruses are effective at transmitting their genetic material. Therefore some groups stand out among the viral vectors, including the retroviruses, adenoviruses, adeno-associated viruses and herpes simplex virus. In transfection, the plasmid with the therapeutic gene can be incorporated to the target cell by physical (electroporation, biobalistic process, sonoporation, hydrodynamics and magnetofection), chemical (lipoplex, polyplex), or biological (infection via bacteria, virosome, Ad-lipo) methods. (Table 1) The choice of the current vector is based on the type of disease, the size of the therapeutic gene, the therapy route and the duration of the gene expression; these characteristics are important for the gene transfer to occur successfully.[9,11,12]

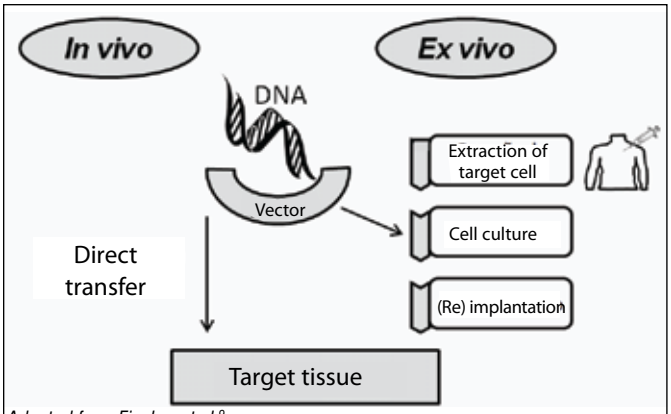
Moreover, the therapeutic gene can be delivered to the site of interest by the in vivo strategy, in which the gene is transferred directly to the site. This type of strategy has proved to be simple, low costing and minimally invasive. On the other hand, in the ex vivo strategythe transfer of the gene of interest is performed in the cells that were collected from the patient, followed by re (implantation) in the target tissue. (Figure 1) For this reason, this kind of approach is more meticulous and expensive.[9]

Each strategy has advantages and disadvantages. While in the in vivo strategy the material appears to be restricted to the injected site and to present low efficiency in transduction, in the ex vivo strategy this efficiency is greater, with the possibility of controlling viral particles and plasmids, yet this process lasts longer.[9,11,12]

This review aims to focus on the most relevant studies on gene therapy for osteoporosis treatment developed in recent years in animal models, as well as the future prospects of this therapy.

Table 1. *Main types of vector used in gene therapy to potentiate the entrance of DNA into the nucleus of the target cell.*

Types of vector		
Viral	**Non-viral**	
Retrovirus	Naked DNA	
Adenovirus	**Physical:**	
Adeno-associated		Electroporation
Herpes simplex virus		Biobalistic process
		Sonoporation
		Hydrodynamics
		Magnetofection
	Chemical:	
		Lipoplex
		Polyplex
	Biological:	
		Bacteria
		Virosome
		Ad-lipo



Adapted from Fischer et al.[9]

Figure 1. *Diagram of the delivery strategies of the therapeutic gene to the site of interest. In vivo transduction involves the direct transfer of the gene and ex vivo transduction involves the collection of the target cell; expansion in cell culture, transduction of the vector and (re)implantation at the site of interest.*

The methodology used was a review of literature carried out by electronic search in the *MEDLINE/PubMed*, LILACS, ISI -*Web of Science* and SciELO databases with special reference to the original articles involving the key words *osteoporosis, gene therapy, viral vector, non-viral vector, adenovirus, retrovirus, adeno-associated virus, lentivirus, bone morphogenetic protein and osteoprotegerin*, using as a reference either MeSH (*Medical Subject Headings*) or DeCS (Health Sciences Descriptors). The inclusion criteria were studies published in the English, Portuguese and Spanish languages in recent years. The main experimental articles are expressed in Table 2 and some of these were cited in the text.

Physiopathology of osteoporosis

The human skeleton is constantly renewed by the joint and coordinated action of the osteoblasts and osteoclasts, specialized cell types, responsible for bone remodeling. The imbalance between the formative activity of the osteoblasts and resorptive activity of the osteoclasts results in loss of bone mass and skeletal fragility, contributing to the physiopathology of osteoporosis.[2] Parathormone (PTH) and calcitonin are the main regulators of calcium homeostasis and respond to the changes in calcemia. The reduction of calcemia induces an increase in PTH production, while rising values decrease its production. The presence of G protein-coupled type 1 PTH receptor (PTHR1) in the osteoblast enables the recognition of the amino-terminal portion of the PTH, which results in activation of the protein kinase C (PKC) routes and the protein kinase A route (PKA). Although the two routes are activated by PTH, PKA is the main route of activation,[8] leading to the increase in the number of osteoclasts. The simplified mechanism is shown in Figure 2.

Unlike PTH, calcitonin acts by decreasing the level of serum calcium by stimulating osteoblastic activity.[2]

Multifactorial causes can be attributed to the triggering of osteoporosis, such as decrease in the ingestion or intestinal ab-

Table 2. *Main gene therapy studies conducted for osteoporosis treatment in non-human animal models in recent years.*

Experimental studies of gene therapy for osteoporosis

Therapeutic Gene	Vector	Quantity	Model	Investigator	Year
CXCR4 and Cbfa-1	MSC– mediated Adenovirus	1x10 ^{6} cells	Mice♀	Lien *et al.*[20]	2009
BMP-2	MSC– mediated Adenovirus (Ad5F35)	2500 viral particles	Mice♂	Gugala *et al.*[24]	2007
RANK-Fc	MSC–mediated Retrovirus	1x10^{7} cells per mL (total volume of 0.2mL/animal)	Mice♀	Kim *et al.*[18]	2006
BMP-2	Naked plasmid	25 µg	Rats♂	Kawai *et al.*[23]	2005
Human β3 integrin	(Mononuclear cells– mediated Retrovirus (Lentivirus	2x10 ^{6} mononuclear cells	Mice♀	Zhao *et al.*[31]	2005
BMP-2	MSC–mediated Adenovirus (Ad5)	1x10^{7}and 2 x10^{7} cells per block	Rats♂	Yue *et al.*[22]	2005
hOPG	Adeno-associated virus (AAV-2)	5x10 ^{9}viral particles	Female Rats♀	Ulrich-Vinther *et al.*[26]	2005
hPTH and RGD	Naked plasmid	300 µg of RGD and100 µg of hPTH	Female Rats♀	Chen *et al.*[16]	2005
hOPG	Adeno-associated virus (AAV-2)	6x10^{11}viral particles	Mice♀	Kostenuik *et al.*[27]	2004
Preand prep-prosequence of hPTH	Naked plasmid	50 µg	Mice♀	Liu *et al.*[33]	2004
hPTH	Nakied plasmid	50 µg	Mice♀	Liu *et al.*[17]	2003
BMP-4	Adeno-associated virus (AAV-2)	2x10^{12}viral particles	Rats♂	Luk *et al.*[21]	2003
IL-1Ra	Adenovirus (Ad5)	1x10^{10}viral particles	Mice♀	Baltzer *et al.*[14]	2001
BMP-2	Cells–mediated Retrovirus	5x10 ^{6} cells	Mice♂	Engstrand *et al.*[25]	2000

sorption of calcium and vitamin D, duration of the reproductive cycle, genetic factors, habits such as alcohol and tobacco, exercise and others.[13]

In spite of efforts, the physiopathological mechanisms involved in the osteoporosis process remain incomplete.

Gene therapy: pre-clinical studies

One of the first studies to evaluate the possibility of using a murine model for gene therapy for the treatment of osteoporosis was developed by Baltzer *et al*.[14] In this study the authors used a model of oophorectomized female mice and the adenovirus serotype 5 vector to perform the delivery of the human IL-1Ra gene in the medullary cavity of the femoral bone. The IL-1Ra gene is responsible for encoding the "interleukin-1 receptor antagonist" protein. The biological function of this protein is reversed to one of the activities of the inflammatory cytokine IL-1 (Interleukin-1), which is a bone resorption inducer and is increased in postmenopausal women. In this manner, a protein that inhibits IL-1 activity appears to be an important tool in the attempt to decrease bone loss. The results showed that all the bones were protected against loss of bone mass, and not just the femur that received the injection with the vector. The authors reinforce the idea proposed by Oyama *et al*.[15] that transduced cells are capable of moving through the bone marrow of different bones.

Chen *et al*.[16] also used the oophorectomized animal model to induce bone loss, but used female rats at an age of 56 weeks. After the surgical procedure the quadriceps muscle of both legs was transfected with the naked plasmid containing the PTH gene or the peptide-treated RGD encoding gene. After

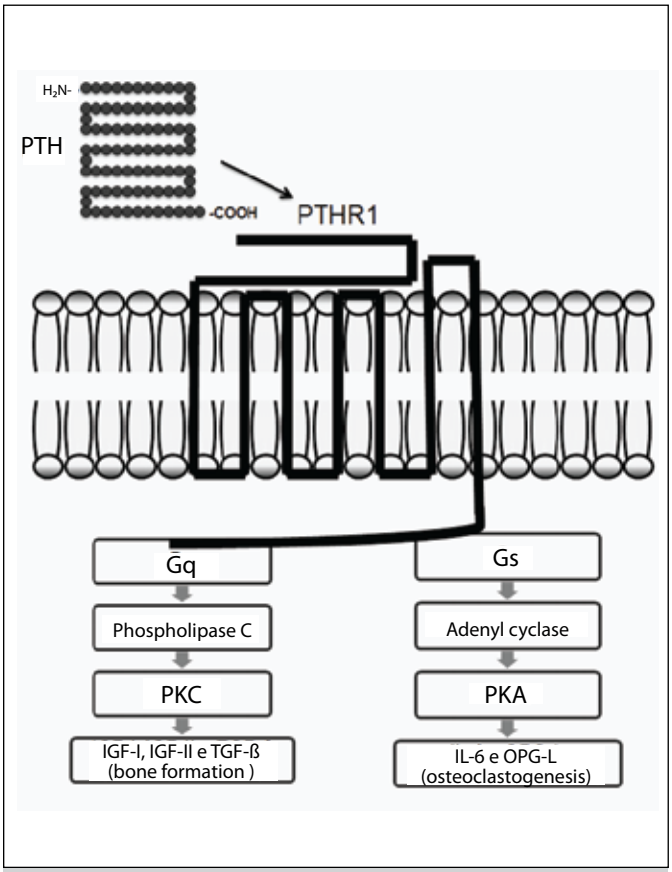


Figure 2. *Diagramof interaction between PTH with the receptor PTHR1 present in the osteoblasts.*

three months the animals were sacrificed and the results showed a 10.1% increase in bone mineral density in the group that received the PTH gene and an 8.9% increase in the one that received the RGD gene. Bone loss was avoided in the animals that received the RGD gene, since this is an antagonist of some integrins responsible for cell adhesion, such as human β3 integrin. Therefore the osteoclasts are unable to maintain their adherence to the cell surface, and the resorption rate decreases, besides which this molecule also induces osteoclast apoptosis. Now in the animals that received the PTH encoding gene, the increase in the parameters evaluated can be explained by the fact that PTH exercises anabolic action when administered intermittently and in low doses.[8]

In a previous study conducted by Liu *et al*.[17], the authors had already successfully performed the transfection of the quadriceps muscle of female mice with PTH encoding gene. On that occasion they had observed that at the end of eight weeks of treatment the expression remained. In order to increase transfection efficiency the authors used the method known as electroporation. In this method an electrical discharge was applied using a pistol, bringing about a change in the permeability of the plasma membrane, which facilitated the penetration of the gene in the target cells.[12]

Many researchers use the mesenchymal stem cells (MSCs) as mediators for osteoporosis treatment, inducing their differentiation in osteoblasts and avoiding accentuated bone loss.[18,19]

The first study that showed the applicability of the systemic transplantation of genetically manipulated and intravenously injected MSCs is recent. After inducing bone loss with glucocorticoids, the authors transduced MSCs with the adenovirus carrying the encoding gene of the CXCR4 or CXCR4 protein with Cbfa-1 in female mice, and noticed that the total recovery of bone mass occurred in the animals that expressed the CXCR4 protein while strength and hardness was recovered only in the group that received the two genes. The recovery of bone mass occurred due to the fact that the cells transduced with CXCR4 played a crucial role in osteogenic differentiation.[20]

With the aging of the individual, the number of MSCs decreases, but the differentiation capacity does not change, which could represent a problem, since the studies use relatively young oophorectomized animal models in comparison with what occurs in type I primary osteoporosis[18] (characterized by estrogen deficiency in postmenopausal women).

In view of the foregoing, there is an evident need for more studies focusing on the quantity of MSCs and the differentiation capacity during the different stages of human development, particularly after menopause.

Most studies in gene therapy for osteoporosis employ the growth factors known as BMPs (bone morphogenetic proteins). These proteins are part of the TNF-ß (tumoral necrosis factor ß) superfamily, and act in differentiation, proliferation and chemotaxis, stimulating osteogenesis in animals and humans. The BMPs most frequently studied and employed in gene therapy include BMP-2 and BMP-4.[21-24]

Yue *et al*.[22] showed that the quantity of MSCs diminishes with age, yet the BMP-2 gene expression by MSC does not change when injected in animals of different ages, and in fact stimulates bone formation in elderly animals. To study the repair of fractures, the same authors perforated the femoral bone then applied a porous block of calcium phosphate on the perforation. Before being applied on the site, this block was cultivated for seven days with MSCs transduced with the adenoviral vector (Ad5) containing the BMP-2 gene. After 6 weeks the MSCs expressed BMP-2. The data showed that the BMP-2 gene restored osteogenic potential to the MSCs. The authors reinforce the idea that the incorporation of genetic material ex vivo or in vivo by means of the MSCs is a useful approach to the prevention or treatment of osteoporotic fractures.

Kawai *et al*.[23] demonstrated that the plasmid transporting the BMP-2 gene, besides inducing endochondral ossification, was also able to induce intramembranous ossification in the gastrocnemius muscle of rats with the aid of transcutaneous electroporation.

The adeno-associated,[21] adenovirus,[22,24] retrovirus[25] and naked plasmid[23] vectors have already been used for the successful transfer of BMP genes. There was a positive response in the bone tissue in all of the above-mentioned vectors, but the choice of the best vector for osteoporosis treatment is still under discussion.

Besides the possibility of treating osteoporosis by stimulating bone formation, gene therapy can also act in the resorption mechanisms.[26-28] Osteoprotegerin (OPG) is an endogenous protein synthesized by the osteoblasts, and is responsible for the inhibition of osteoclastogenesis and of osteoclast activity. OPG acts as an attraction to RANKL (receptor activator of nuclear factor-kB ligand), impairing its bond with RANK (receptor activator of nuclear factor-kB), for which reason there is no induction of the intracellular signals responsible for the differentiation, function and survival of the osteoclasts. OPG production is regulated by molecules that increase its synthesis (IL-1a, IL-6, IL-11, IL-17, IL-18, TNF-α, TNF-ß, BMP-2, estrogen, calcium and vitamin D3) and others that decrease it (parathormone, glucocorticoids and prostaglandin E2).[29]

Kostenuik *et al*.[27] managed to stabilize bone loss in the initial process of osteopenia, in the model of oophorectomized female mice. The authors used the adeno-associated vector as a transporter of the human OPG gene. The bone mineral density of the tibia increased significantly in the animals that received the OPG gene, with a reduction also in the quantity of osteoclasts on the surface of the proximal tibial metaphysis. The sham-operated mice (exposure of the ovaries, but without removal), also exhibited an increase of bone mineral density when compared with the other animals that received a gene without effect on the bone metabolism.

The adenovirus carrying the OPG gene was also studied for osteoporosis treatment. In transducing a host cell, this type of vector maintains the genome not integrated into the chromosome (episomal form), but when the infected cell divides the episome may get lost, which can also lead to an immune response, and consequently the effect of the therapeutic gene is lost. Nevertheless, Bolon et al.[28] demonstrated that the expression can be maintained for

at least 18 months and lead to an increase of bone density. The interleukins represent an important target for osteoporosis treatment and prevention. It was demonstrated that IL-11 is an important osteoblastogenesis and bone formation stimulating factor, possibly as it increases the production of BMPs. IL-11 may represent a new therapeutic strategy for the treatment of the senile osteoporosis.[29]

It is known that estrogen deficiency causes an increase in IL-7 production, reflected in a greater quantity of osteoclasts. Thus the neutralization of IL-7 has an effect on the diminishment of bone resorption. The role of interleukins in bone metabolism has been increasingly investigated, and many of the discoveries may help to expose new targets for gene therapy.[30]

CONCLUSION

Few reports in scientific literature used gene therapy as an instrument to treat osteoporosis.

The calcified matrix of the bone tissue represents an additional obstacle in the study of gene therapy for osteometabolic diseases, as the diffusion of the vector through matrix is practically impossible, which makes this therapy more elaborate for osteoporosis, directly affecting the success of the transfection or transduction of the osteogenic cells. One alternative to circumvent this obstacle is related to the use of mesenchymal stem cells carrying the therapeutic gene, since they are osteoblast precursor cells and are reflected in the increase of bone mass due to matrix synthesis.[18,22] Nevertheless, the studies reported reestablished bone mass and thus reduced the risk of fractures.

Most studies reported the use of only one therapeutic gene. Theoretically, nothing prevents the plasmid expression cassette from having genic sequences to act both in the osteoblasts, stimulating bone formation, and in the osteoclasts, decreasing the resorption rate. Thus is possible to potentiate the treatment for osteoporosis and to have a faster positive response, particularly for individuals with severe osteoporosis.

A large portion of the studies uses OPG, BMP and PTH as promising molecules for osteoporosis treatment, yet there is a lack of new molecules as possible targets to be studied, practically limiting researchers to the use of the same molecules. Nonetheless, the RGD sequence and the study with human ß3 integrin proved to be important targets for antiresorptive gene therapy.[16,31]

Future studies should be carried out with a focus on the use of large animal models, the use of new therapeutic genes and of the best vector for gene transfer.

The cost, route of administration and side effects are some of the undesirable factors that may culminate in the abandonment or interruption of the conventional treatment, compromising the efficacy of the medication and the patient's health.[8] In patient treated by gene therapy, the abandonment and/or interruption of treatment is expected to become less frequent.

In the attempt to reduce negative aspects in the current therapeutic approach to osteoporosis, gene therapy shows itself to be an important tool. It is expected that in the future, by means of one or few minimally invasive interventions, the therapeutic gene will be able to reestablish bone tissue function and to cause few or no side effects. For this reason, control of the expression level is essential, since a low level may not produce the therapeutic results and the inverse may be harmful to the cell. Control mechanisms are being studied to control gene expression.[11,32]

With the advances of genetic engineering techniques, the cost of this therapy should drop considerably. The benefits that this tool will be able to bring to patients in the future should contribute substantially to the increase in the prospects and in the quality of life of the elderly, particularly of the female population, which suffers most from this disease.

The prevention or the faster reestablishment of fractures arising from osteoporosis will certainly entail a lesser economic impact on the government budget.[5]

In the near future, clinical protocols involving humans should bring hope to thousands of people that suffer from this disease.

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